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## **Alternative Splicing and SR proteins in ABA-mediated Stress Responses**

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## ABSTRACT

Absciscic acid (ABA), the major plant stress hormone, plays a crucial role in the response to the two most pervasive causes of loss of crop productivity worldwide, drought and high salinity.

To adapt to an environment in constant change, plants, as sessile organisms, have evolved high degrees of developmental plasticity, which is ultimately regulated at the genome level. The exceptional versatility associated with gene regulation by alternative splicing is likely to play a prominent role in plant stress responses. By being alternatively spliced, a single gene can generate multiple transcripts, and eventually more than one polypeptide, upscaling the genome's coding capacity, thus generating proteome diversity and functional complexity.

Serine/arginine-rich (SR) proteins are a conserved family of RNA-binding proteins that act as major modulators of alternative splicing. These RNA-binding proteins influence splice site selection in a concentration and phosphorylation-dependent manner and are known to be key players in mammalian alternative splicing. While their functional relevance in plants remains largely unknown, mounting evidence suggests a central role for these proteins in the response to stress, particularly by targeting the ABA pathway. Consistently, the expression of nearly half of the *Arabidopsis thaliana* SR genes is regulated by this phytohormone.

The work presented in this thesis sheds light on the functional significance of the *Arabidopsis* *SR34* and *SCL33*, two uncharacterized ABA-responsive SR genes. Phenotypical analyses of the corresponding *sr34-1* and *sc/33-1* mutants show that both of these genes play important roles in regulating ABA-related stress responses during early plant development. While the *sc/33-1* mutant does not appear to be impaired in stress responses, the loss of *SCL33* function in the *sc/30a-1* mutant rescues the latter's stress hypersensitive phenotype, suggesting that *SCL33* and *SCL30a* have opposite functions in a same pathway controlling ABA-related stress responses. On the other hand, the *sr34-1* mutant, in which *SR34* expression is strongly downregulated, shows hypersensitivity to ABA, high salinity, and drought during seed germination, pointing to a role for this splicing factor as a negative regulator of ABA-mediated stress responses. Moreover, here we show that this gene generates at least three splice variants, for which the subcellular localization of the corresponding protein products was assessed by two different approaches. While in *Nicotiana benthamiana* transient assays all *SR34* splice forms were localized in the nucleus, both a nuclear and cytoplasmic localization was detected in *Arabidopsis* transgenic root tissues.

Together, the results gathered during the execution of this work provide additional evidences for a key role of SR proteins in the regulation of plant stress responses.

**KEYWORDS:** Abiotic stress; Absciscic acid; Alternative Splicing; *Arabidopsis thaliana*; SR Proteins.

## RESUMO ALARGADO

Condições ambientais de stress, tais como a seca, a elevada salinidade e temperaturas extremas, constituem as causas mais comuns de perda de produtividade agrícola a nível mundial. Estes tipos de stress resultam em stress osmótico, sendo caracterizados pela redução da água disponível que a planta consegue utilizar.

Não possuindo capacidade de locomoção, as plantas desenvolveram estratégias adaptativas únicas, quer a nível de desenvolvimento quer a nível fisiológico, que lhes permitem fazer face a um ambiente em constante mudança. As hormonas vegetais têm um papel crucial nesta resposta, atuando não só como reguladores do crescimento e desenvolvimento vegetal, mas também como mediadores da resposta ao stress. Dentro das diferentes fitohormonas, destaca-se o ácido abscísico (ABA). Em resposta ao stress osmótico é promovida na planta a síntese desta hormona, que será responsável pela ativação/repressão de diversas cascatas de sinalização, regulando numa última fase alterações na expressão génica de modo a tolerar ou combater o stress. Esta hormona é igualmente responsável pela regulação da maturação e do estado de dormência das sementes, atuando posteriormente no desenvolvimento do embrião e na sua germinação. Em condições de stress, o ABA reprime a germinação e, em estados mais tardios do desenvolvimento, é responsável pelo controlo da abertura estomática, prevenindo perdas excessivas de água por transpiração. O estudo das vias de sinalização dependentes desta hormona tem-se intensificado nos últimos anos, existindo cada vez mais evidências que apontam para uma ligação entre as respostas ao stress dependentes do ABA e o processamento do mARN através do mecanismo de *splicing*.

A maioria dos genes dos organismos eucariotas contém regiões codificantes, denominadas exões, interrompidas por regiões não-codificantes, os intrões. O processo pelo qual os intrões são removidos do mARN precursor (pré-mARN) e os exões ligados entre si denomina-se *splicing*. Múltiplas formas de mARN maduro podem ser obtidas a partir de um único pré-mARN através de um mecanismo conhecido por *splicing* alternativo. Este processo permite a produção de mais do que uma proteína a partir de um único gene, contribuindo assim decisivamente para a criação de diversidade transcritómica e proteómica. Por outro lado, este mecanismo proporciona uma forma rápida e versátil de regular a expressão génica, o que pode desempenhar um papel importante nos processos de adaptação das plantas. Nos últimos anos, o número de exemplos de *splicing* alternativo descrito em plantas tem aumentado exponencialmente, havendo evidências crescentes de que este mecanismo de regulação pós-transcricional desempenha um papel fundamental na resposta das plantas ao stress ambiental.

As proteínas SR, ricas em serina e arginina, constituem uma família altamente conservada de fatores de *splicing*, presentes tanto em metazoários como em plantas. Caracterizam-se estruturalmente por possuírem um ou dois domínios de reconhecimento do ARN (RRM) e um domínio rico em dipéptidos de serina e arginina (RS), responsável por interações proteína-proteína. Em

comparação com os animais, as plantas possuem um maior número de proteínas SR, em parte devido a duplicação gênica intercromossômica. Não se sabe ainda se os genes parálogos resultantes destas duplicações possuem funções redundantes ou se são capazes de desempenhar papéis diferentes, tendo sido apenas demonstrado que alguns destes pares de parálogos possuem perfis de expressão diferentes durante o desenvolvimento vegetal. No genoma de *Arabidopsis thaliana* existem vinte genes codificando proteínas SR ou semelhantes, seis dos quais possuem um gene parálogo.

As proteínas SR são essenciais na regulação tanto do *splicing* constitutivo como no *splicing* alternativo, desempenhando um papel-chave nas etapas iniciais de montagem do spliceossoma, influenciando a escolha dos sítios de *splicing* em função da sua concentração e do seu nível de fosforilação. Os genes que codificam estas proteínas são eles próprios alvos de *splicing* alternativo, tornando possível aumentar em seis vezes a complexidade do seu transcrito. Todavia, em *Arabidopsis thaliana*, a maioria destas variantes de *splicing* contém codões de terminação prematuros, codificando proteínas incapazes de realizar a sua função e que constituem alvos para degradação celular.

Existem poucos estudos disponíveis visando a caracterização de proteínas SR em plantas. No entanto, dados recentes apontam para estas proteínas como principais coordenadoras na resposta ao stress, tendo como alvo a via de sinalização do ABA. Um exemplo é o caso da proteína SCL30a, pertencente à subfamília SCL de proteínas SR de *Arabidopsis thaliana*. O laboratório de Biologia Molecular de Plantas, onde o trabalho desta tese foi desenvolvido, descobriu recentemente que um mutante para o gene *SCL30a* de *Arabidopsis* apresenta hipersensibilidade a condições de stress osmótico durante a germinação da semente, e que esta resposta é dependente da hormona ABA. Por outro lado, o mesmo laboratório reportou que o tratamento com ABA exógeno e/ou a alteração dos níveis de expressão de componentes essenciais à síntese ou sinalização desta hormona alteram significativamente a expressão de oito genes codificando proteínas SR em *Arabidopsis*.

O presente trabalho foi iniciado com o objetivo de compreender o papel biológico do *splicing* alternativo no crescimento e desenvolvimento das plantas, utilizando *Arabidopsis thaliana* como organismo modelo. Neste estudo, através da utilização de genética reversa e análises de localização subcelular, investigámos as funções *in vivo* de duas proteínas SR de *Arabidopsis*: a SR34, pertencente à subfamília SR, e a SCL33, um parálogo da SCL30a, ambas pertencentes à subfamília SCL. Os níveis de expressão destas dois genes SR, SR34 e SCL33, são regulados pelo ABA e, no presente trabalho, fornecem-se evidências de que de facto estes dois fatores de *splicing* desempenham um papel nas respostas ao stress mediadas por esta fitohormona.

Este estudo teve início com a caracterização funcional do gene SR34, que se descobriu gerar pelo menos três variantes de *splicing* alternativo. Um mutante com níveis de expressão deste gene fortemente reduzidos mostrou ser hipersensível durante a germinação da semente em condições de

stress cuja resposta é mediada pela hormona ABA. Este resultado preliminar sugere que o factor de *splicing* SR34 constitui um regulador negativo da resposta ao stress durante a germinação. Adicionalmente, por análise da localização subcelular das três isoformas de *splicing* da SR34, concluímos que o *splicing* alternativo deste gene não influencia a localização subcelular da proteína que codifica. Quando expressas de forma transiente em *N. benthamiana*, todas as isoformas de *splicing* apresentaram uma estrita localização nuclear, substanciando o papel desta proteína no processo de *splicing*. Surpreendentemente, quando expressas de forma constitutiva em *Arabidopsis thaliana*, a sua localização não se limitou apenas ao núcleo celular, tendo sido observadas igualmente no citoplasma das células, sugerindo um papel mais generalizado para estas proteínas.

Por outro lado, este trabalho teve como objetivo caracterizar funcionalmente o gene *SCL33* e compreender uma possível interação genética com o seu parólogo *SCL30a*. Por análise de um conjunto de mutantes onde a expressão destes genes se encontra afetada, demonstrámos que por si só o gene *SCL33* não parece estar envolvido na resposta a condições de stress durante a germinação da semente ou durante o desenvolvimento vegetal precoce. No entanto, a utilização de um duplo mutante para estes genes permitiu verificar que, na ausência de ambos, o fenótipo descrito para o mutante do gene *SCL30a* é resgatado, sugerindo que as proteínas SCL30a e SCL33 atuam de forma antagónica na mesma via de sinalização de resposta a stress mediada pela hormona ABA.

Tanto para os genes *SCL33* e *SCL30a*, como para o SR34, observou-se uma expressão generalizada dos seus transcritos em diferentes órgãos e ao longo do desenvolvimento vegetal. Estes resultados poderão assim sugerir um papel para estas proteínas para além da fase precoce do desenvolvimento. No entanto, ainda que globalmente expressos, este estudo demonstra que mutantes para estas proteínas não apresentam defeitos, comparativamente ao controlo, na regulação da abertura estomática.

Em suma, o trabalho aqui apresentado fornece uma importante contribuição para a elucidação do papel das proteínas SR, e consequentemente da relevância biológica do *splicing* alternativo, na resposta das plantas ao stress, durante a germinação da semente. Adicionalmente, aqui reportamos uma expressão generalizada para estas proteínas SR, sugerindo que estes fatores de *splicing* poderão regular variados processos celulares ao longo dos diferentes estágios de desenvolvimento vegetal, não estando a sua função limitada ao processo de germinação da semente ou a condições de stress.

#### **Palavras-chave:**

Ácido abscísico; *Arabidopsis thaliana*; Proteínas SR; *Splicing* alternativo; Stress abiótico;

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## ABBREVIATIONS LIST

<b>ABA</b>	Absciscic acid
<b>AS</b>	Alternative splicing
<b>bp</b>	Base pair
<b>DNA</b>	Deoxyribonucleic acid
<b>eGFP</b>	Enhanced green fluorescent protein
<b>ESE</b>	Exonic splicing enhancer
<b>ESS</b>	Exonic splicing silencer
<b>hnRNP</b>	Heterogeneous ribonucleoprotein
<b>ISE</b>	Intronic splicing enhancer
<b>ISS</b>	Intronic splicing silencer
<b>KO</b>	Knockout
<b>LB</b>	Left border
<b>mRNA</b>	Messenger RNA
<b>Pre-mRNA</b>	Precursor mRNA
<b>PTC</b>	Premature termination codon
<b>NMD</b>	Nonsense-mediated decay
<b>RNA</b>	Ribonucleic acid
<b>RNP</b>	Ribonucleoprotein
<b>RRM</b>	RNA-recognition motif
<b>RS</b>	Arginine/serine-rich
<b>snRNP</b>	Small nuclear ribonucleoprotein
<b>SR protein</b>	Serine/arginine-rich protein
<b>TAIR</b>	The <i>Arabidopsis</i> Information Resource
<b>UTR</b>	Untranslated region
<b>WT</b>	Wild type

## **I. INTRODUCTION**

### **1. Abiotic stress**

Abiotic stresses such as drought, high salinity and extreme temperatures represent the most pervasive causes of reduced crop productivity worldwide. Therefore, a major goal in plant science is to understand how plants respond to and withstand environmental stresses successfully. Abiotic stresses trigger many biochemical, molecular and physiological alterations as well as responses that influence various cellular and whole-plant processes [reviewed in 1]. Drought, high salinity, and temperature stress lead to reduced water availability (also known as osmotic stress), characterized by a decreased turgor pressure and water loss. Osmotic stress promotes the synthesis of the plant stress hormone abscisic acid (ABA), which then triggers major changes in gene expression and adaptive physiological responses [reviewed in 2]. ABA coordinates several signal transduction pathways involved in the above-mentioned abiotic stresses, all of which are known to increase endogenous levels of the phytohormone. In seeds, ABA regulates embryo and seed development, dormancy establishment and the transition to germination [reviewed in 3, 4]. Under stress conditions, ABA blocks seed germination and, later during development, it regulates stomatal aperture, thus preventing excessive water loss from leaves [reviewed in 5, 6]. Moreover, genetic studies on *Arabidopsis* mutants exhibiting an abnormal response to abiotic stress or ABA revealed a clear link between mRNA processing/metabolism and ABA-related stress responses [reviewed in 7]. Importantly, several lines of evidence point to a major role for a posttranscriptional regulatory mechanism called alternative splicing (AS) during plant stress responses. Indeed, plant genes associated with various stresses are particularly prone to AS [8, 9], which in plants is also markedly affected by abiotic stress. An important link between AS and plant responses to ABA stresses has been provided recently with the discovery that HAB1, a type 2c phosphatase, known as a central regulator of the ABA signaling pathway, is alternatively spliced in response to ABA [10]. The ratio between the two generated transcripts is an important on-off switch in ABA signaling and plant responses to ABA.

### **2. Pre-mRNA splicing in plants**

The majority of protein-coding genes in photosynthetic eukaryotic organisms are discontinuous, with coding sequences (exons) disrupted by noncoding regions (introns). Splicing is a fundamental posttranscriptional gene regulation process that removes the introns from the precursor messenger RNA (pre-mRNA) and joins the exons in order to obtain a mature mRNA that may later be translated into a protein [11, 12]. While the functional significance of pre-mRNA splicing in animal systems has been well-established, mounting evidence suggests a crucial role for this mechanism in plants as well,

where it acts during photosynthesis, defense responses, flowering, circadian clock control, as well as in hormone signaling, among other functions [13, 14].

The RNA cleavage and ligation reactions necessary for intron removal in protein-coding mRNAs (and long noncoding RNAs) occur in a multi-megadalton ribonucleoprotein (RNP) complex, called the spliceosome. Both the conformation and composition of the spliceosome are highly dynamic, allowing the splicing machinery to be both accurate and flexible. In plants as in animals, there are two types of spliceosomes. The more abundant type is called the U2-dependent spliceosome and performs splicing of U2-dependent introns, while the less abundant U12-dependent spliceosome is only present in a subset of eukaryotes and splices the rare U12-type class of introns. Both spliceosomes consist of a large number of non-small nuclear ribonucleoprotein (non-snRNP) and five small nuclear ribonucleoproteins (snRNPs), U1, U2, U5 and U4/U6 in the major spliceosome, and U11, U12, U5, U4atac/ U6atac in the minor spliceosome [reviewed in 15].

For accurate splicing of the pre-mRNA in higher organisms, the assembly of the spliceosome on introns in pre-mRNAs is also governed by different splicing signals. First, introns are delimited by short consensus sequences: the GU and AG, respectively at the 5' and 3' splice sites, which mark the border between exons and introns; the branch point, a sequence near the 3' splice site that is defined by an adenosine residue; and the polypyrimidine tract (Py), a stretch of pyrimidines downstream of the branch point site [16].

The use of the splice sites is also regulated by *cis*-regulatory sequences and *trans*-acting factors. The former include moderately conserved, short consensus sequences known as exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs), which differ in their locations on the pre-mRNA and in the way they affect the usage of a splice site [17]. These elements function by recruiting additional RNA-binding proteins during the assembly and the catalytic cycle of the spliceosome. Strong splice sites (i.e., those that are more similar to the consensus sequence) are more efficiently recognized and used than weak or suboptimal splice sites. Moreover, *trans*-acting factors, which include members of the well-characterized serine-arginine (SR) and heterogeneous nuclear ribonucleoprotein (hnRNP) families of proteins, as well as tissue-specific factors, work through binding to these splicing enhancers and silencers, affecting splice site selection [reviewed in 17].

The splicing reaction proceeds via two sequential transesterification reactions. In the first reaction, the 2' hydroxyl group of an intronic adenosine residue (branch-point site) carries out a nucleophilic attack on the phosphate group between the 5' exon and the intron, generating a phosphodiester bond and, consequently, forming an intermediate looped structure known as lariat, and leaving a free 3' hydroxyl group at the 5' exon. In the second step, the free 3' hydroxyl group of the 5' exon attacks the

phosphate group between the intron and the 3' exon, splicing the two exons together and releasing the intron lariat [reviewed in 18].

In splicing, we may consider two classes of events: constitutive splicing events, where the splice sites are recognized efficiently by the spliceosome and each pre-mRNA from a given gene is spliced in the same way, and AS events, in which recognition and joining of a 5' and 3' splice site pair are in competition with at least one other 5' or 3' splice site, allowing different rearrangements of the gene's coding fragments and resulting in the generation of multiple forms of mature mRNA from the same pre-mRNA molecule [reviewed in 19]. By being alternatively spliced, a single gene can lead to the production of more than one polypeptide, upscaling the genome's coding capacity. One of the most fascinating example of this is the *DSCAM* (*DOWN SYNDROME CELL ADHESION MOLECULE*) gene from *Drosophila melanogaster* that encodes more than 38000 mRNA variants through a complex pattern of AS in four different regions of its pre-mRNA [reviewed in 20]. Consequently, AS of one given gene can lead to the synthesis of numerous proteins with different sequences and domain arrangement and hence potentially different subcellular localization, stability or function. In addition, splicing can also regulate transcript levels by introducing premature termination codons (PTCs) that then target the mRNAs for degradation by nonsense-mediated decay (NMD), a surveillance mechanism that prevents accumulation of truncated and potentially harmful proteins.

More than 95% of human genes are estimated to undergo alternative splicing [21]. In plants, recent high coverage RNA-seq transcriptome analyses estimated that, under control conditions, at least 61% of intron-containing genes of *Arabidopsis thaliana* [22], 63% of soybean (*Glycine max*) [23], 48% of rice (*Oryza sativa*) [24], 40% of maize (*Zea mays*) [25] and 30% of grape (*Vitis vinifera*) [26] undergo AS. Furthermore, the generation of new data from RNA-seq in specific plant-tissues, cell types, developmental stages, or from plants grown under different biotic or abiotic stresses, will likely result in higher rates of alternatively spliced genes in plants. [reviewed in 27].

Most AS events can be classified into four basic types – exon skipping or inclusion, selection of alternative 5' or 3' splice sites and intron retention. In *Arabidopsis*, intron retention is the most prominent alternative splicing event ( $\approx 40\%$ ), followed by alternative 3' splice sites and 5' splice sites, while exon skipping events are relatively rare. By contrast, in humans, intron retention is the least frequent ( $<5\%$ ) event and exon skipping is the most prevalent ( $>40\%$ ) [reviewed in 28].

### 3. SR proteins

SR (serine/arginine-rich) proteins constitute one of the most highly conserved families of splicing factors that are essential for the execution and regulation of constitutive and alternative pre-mRNA splicing. These non-small nuclear ribonucleoproteins (non-snRNP) proteins were originally classified in animals as a family based on their ability to restore splicing activity to splicing factor deficient cell

extracts and on their recognition by the monoclonal antibody mAb104 which recognizes a common epitope enriched in serine (S) and arginine (R) residues in their C-terminus [29, 30].

SR proteins are present in both metazoans and plants, and are structurally defined as a family of RNA-binding proteins with a modular domain structure consisting of one to two amino-terminal RNA recognition motifs (RRMs) that bind to specific regulatory sequences in the pre-mRNA, and a C-terminal domain rich in serine and arginine dipeptide repeats (the RS domain), that both promotes pre-spliceosome assembly and protein-RNA or protein-protein interactions with other components of the splicing machinery [31-34]. SR proteins can bind to auxiliary *cis*-acting regulatory elements on RNA and interact with spliceosome components such as the U2 Auxiliary Factor (U2AF) and the U1 snRNP [35, 36], directing them to the correct splice sites. Thus, SR proteins play also a major role in AS by affecting splice site selection under different conditions and in a concentration-dependent manner [37-39]. In general, these proteins appear to function as splicing activators. However, binding sites on pre-mRNA for SR proteins and other splicing regulators (e.g., hnRNP family) are typically in close proximity, suggesting that an interplay between activation and repression modulates the frequency of exon inclusion [40, 41].

Reversible phosphorylation of the RS domain by several kinases and phosphatases is a fundamental mechanism in the regulation of SR protein activity, as it determines interactions with other RS-domain-containing and spliceosome-related components [36, 42, 43]. In addition, methylation of arginine residues by protein arginine methyltransferases (PRMTs) may affect the correct positioning, activity and/or mobility of SR proteins [44].

At steady-state, SR proteins accumulate in subnuclear speckles, which are storage, assembly, and/or modification compartments for splicing factors [45]. Surprisingly, co-expression of SR proteins that belong to different subfamilies show distinct population of speckles with little or no co-localization, indicating the existence of various types of speckles that differ in the SR proteins they contain [46]. In animals, these RNA-binding proteins shuttle between the nucleus and the cytoplasm, and this shuttling dynamics is linked to their post-splicing activities during mRNA transport [47], stability, nonsense-mediated decay (NMD) [48] and translation [49, reviewed in 50]. In plants, the subcellular localization of the members of the SR subfamily has been investigated using a fluorescence loss in photobleaching (FLIP) approach [45]. In this study, the authors showed that SR30, SR34 and SR34a, shuttles between the nucleus and the cytoplasm. This result could suggest that, as their mammalian counterparts, these SR proteins in plants are involved in post-splicing activities, although further studies of the SR protein subcellular localization and dynamics are needed to confirm the precise molecular functions of each plant SR protein.

In plants, a multitude of these proteins has been already identified in *Arabidopsis* [51], rice [52], *Brachypodium* [53], maize [54], soybean and others [55]. Based on the recent updated definition for

plant SR proteins [32], the *Oryza sativa* and *Brachypodium* genomes encode 22 and 17 SR proteins, respectively, while *Arabidopsis thaliana* has 18 members grouped into six subfamilies. The *Arabidopsis* SR, RSZ and SC subfamilies include direct orthologs of the mammalian SR splicing factors, while the SCL, RS2Z and RS subfamilies are plant-specific [32, reviewed in 56]. In *Arabidopsis*, proteins of the SR subfamily (orthologs of mammalian SRSF1/SF2/ASF) contain an evolutionary conserved SWQDLKD motif in their second RRM followed by the characteristic SR dipeptides. In contrast, the SC subfamily (orthologs of SRSF2/SC35) contains proteins with a single RRM preceding an RS domain, and the members of the RSZ subfamily (orthologs of mammalian SRSF7/9G8) contain one zinc knuckle between the RRM and the RS domain. The proteins of the plant-specific RS2Z subfamily contain two zinc knuckles and have an additional acidic C-terminal extension rich in serine and proline residues (SP-rich region) following the RS domain, while RS subfamily proteins contain two RRMs (without the SWQDLKD motif) followed by the RS domain rich in RS dipeptides. The plant-specific SCL subfamily is similar to SRSF2 (RRM domain), but has an N-terminal charged extension. Additionally, the *Arabidopsis* genome encodes two SR-like proteins, SR45 and SR45a, which display an atypical domain organization (two RS domains flanking an RRM) that does not strictly meet the currently accepted SR protein nomenclature criteria [32].

Interestingly, several plant SR proteins have paralogs due to whole-genome and segmental duplications. It is not known whether these paralogs are functionally redundant or have evolved to perform non-redundant functions. Nevertheless, over the six *Arabidopsis* SR gene pairs, none of the paralogs show the same expression level during different developmental stages. This suggests that SR paralogs have probably evolved specific functions, mainly by adopting different expression patterns. By contrast, the *Arabidopsis* SR genes that do not have any paralog display overlapping expression patterns [55].

Moreover, SR pre-mRNAs in plants are extensively alternatively spliced [52, 54, 57-59, reviewed in 60]. In *Arabidopsis*, the transcriptome complexity of SR pre-mRNAs is increased by about six fold due to AS. SR proteins have been found to regulate their own splicing and/or that of other splicing factors [52, 57]. Sequence analysis of all SR splice variants from *Arabidopsis* has revealed that over 50 putative proteins can be produced, many of which lack one or more domains. About 55 SR splice variants in *Arabidopsis* contain a PTC, and half of the PTC-containing splice forms were confirmed to be targets for degradation through NMD [61]. It is known that the splicing pattern of some SR genes differs in a developmental and tissue-specific manner and is modified in response to various stresses, suggesting that regulation of AS is important for development and stress responses [60]. Also in support of a functional role for AS and SR proteins in stress responses, plant SR protein genes are stress-regulated at the transcriptional, posttranscriptional and posttranslational levels, with different environmental cues controlling their own AS patterns, phosphorylation status and subcellular distribution [33].

Several lines of evidence point to a role for SR proteins as central coordinators of plant abiotic stress responses, namely by targeting ABA pathway components. First, it was shown that RS40 and RS41, two members of the plant-specific RS subfamily are important regulators of salt and ABA stress responses in *Arabidopsis* (58). Furthermore, work in the Plant Molecular Biology (PMB) laboratory at the *Instituto Gulbenkian de Ciência* (IGC), where the experimental component of this thesis was conducted, has shown that two *Arabidopsis* SR proteins mediate responses to distinct environmental cues by regulating the ABA pathway. A knockout mutant for the *Arabidopsis* SR-like SR45 protein displays a glucose-mediated early growth arrest that is rescued by an ABA biosynthesis inhibitor. Indeed, the mutation confers enhanced ability to accumulate ABA in response to glucose, showing that SR45 negatively regulates sugar signaling by repressing the ABA pathway. On the other hand, the plant-specific *SCL30a* is a negative regulator of salt and drought responses conferring stress tolerance during seed germination. Indeed, the *scl30a-1* loss-of-function mutant shows a hypersensitive phenotype in response to ABA-related stresses. Moreover, the use of ABA biosynthesis inhibitors and epistatic analyses using genes involved in ABA biosynthesis or signaling demonstrate that the control of these stress responses by *SCL30a* is ABA-dependent (Carvalho *et al.*, manuscript in preparation). Interestingly, the *Arabidopsis* *SCL30a* and *SCL33* genes, two members of the plant-specific SCL subfamily, arose from a genomic interchromosomal duplication [51]. Considering that the *scl30a-1* single mutant displays a strong phenotype in response to stress, it is likely that *SCL30a* and *SCL33* adopted at least partially distinct functions. Nevertheless, the functional role of *SCL33* during stress responses remains elusive.

A recent study from the PMB laboratory indicates that expression of eight of the *Arabidopsis* SR and SR-like genes is affected by exogenous ABA and/or by altered levels of key ABA biosynthesis and signaling components [60], including *SCL30a* and *SR45* that have been implicated in ABA responses *in vivo* ([14, 62] and Carvalho *et al.*, manuscript in preparation).

The work presented here aimed at using gene expression and subcellular localization analyses combined with reverse genetics in *Arabidopsis thaliana* to uncover the functional significance of two uncharacterized plant SR genes, the *Arabidopsis* *SR34* and *SCL33*, whose expression is known to be ABA-responsive [60].

We first conducted the functional characterization of the *Arabidopsis* ABA-responsive *SR34* gene, which we have found generates at least three splice variants. A mutant where *SR34* expression is strongly downregulated shows hypersensitivity to ABA-related stresses during germination. These preliminary results point to a role for this splicing factor as an important negative regulator of ABA-mediated stress responses during seed germination. The subcellular localization of the different *SR34* splice forms was assessed in *N. benthamiana* and in *Arabidopsis* transgenic plants. In *N. benthamiana*,



all three SR34 splice forms were localized in the nucleus. This observation was in contrast to their nucleo-cytoplasmic localization pattern observed in *Arabidopsis* root tissues.

Additionally, to characterize and understand the functional redundancy between *SCL33* and *SCL30a*, epistatic analyses were carried out using a double mutant for these two genes. By analyzing the phenotype of the *SCL33* mutant under stress conditions, we found that this gene alone is not involved in the control of plant stress responses during germination. However, the loss of *SCL33* function in the *SCL30a* mutant rescues this mutant's phenotype. The results presented here therefore suggest that *SCL33* and *SCL30a* act antagonically to control plant stress responses. A potential functional relationship between these two genes is discussed.

## II. MATERIALS AND METHODS

### 1. Plant materials and growth conditions

*Arabidopsis thaliana* (L.) Heyhn, ecotype Columbia-0 (Col-0), was used as the wild type in all experiments. Seeds were always first surface-sterilized for 10 min in 50% (v/v) bleach and 0.028% (v/v) Tween 20 (20% v/v) under continuous shaking and then washed six times in sterile water. Following stratification for three days in the dark at 4°C, seeds were sown on Petri dishes containing MS medium: 1 X Murashige & Skoog (MS) salts (Duchefa Biochemie) supplemented with 0.5 mM myo-inositol (Fluka) and 2.5 mM MES (Calbiochem), adjusted to pH 5.7. The plates were then placed in a controlled environment reach-in chamber under long-day conditions — 16 h light (100  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , cool white fluorescent light) at 22°C/8 h dark at 18°C — and 60% relative humidity. After two to three weeks, plants were transferred to soil in individual pots and placed in a growth chamber under the same conditions as described above.

### 2. Isolation of T-DNA insertion mutants

The T-DNA insertion lines SALK\_106067, SALK\_102166, SALKseq\_105967 and later SALK\_010894 for the *SR34* (At1g02840) gene were identified from the *Arabidopsis* insertion mutant library “TDNA-express: *Arabidopsis* gene mapping tool” (<http://signal.salk.edu/cgi-bin/tdnaexpress>) and purchased from the Nottingham *Arabidopsis* Stock Centre (NASC). Seeds from these insertion lines were surface-sterilized as described above and plated on MS medium supplemented with 50  $\mu\text{g}/\text{mL}$  of kanamycin to select for seeds containing the T-DNA insertion. In case of silencing of the kanamycin resistance, half of the seeds were also plated on MS medium without kanamycin. The insertion lines were genotyped by PCR using *SR34*-specific primers flanking the insertion and primers annealing at the left border of the T-DNA (**Supplementary Table 1**). To confirm the exact position of the insertion, PCR

products from homozygous mutants containing the junction between the left border of the T-DNA and the *SR34* genomic region of the insertion were sequenced (AB DNA Sequencer 3130xl).

Homozygous lines for the T-DNA insertion in the *SCL33* (AT1g55310) and *SCL30a* (AT3g13570) genes (SALK\_041849 and SALK\_058566, respectively) had been previously isolated in the PMB laboratory. These two mutant lines were crossed to produce an *sc/30a-1 sc/33-1* double mutant, and homozygous lines for the two insertions were isolated by PCR genotyping as described above.

### **3. RNA Extraction, cDNA Synthesis, and RT-PCR analyses**

Total RNA was extracted from different plant tissues (dry and imbibed seeds, seedlings, roots, rosette leaves, stems, flowers, siliques and senescent leaves) and developmental stages using the innuPREP Plant RNA kit (Analytik Jena BioSolutions) following the manufacturer's protocol. All RNA samples were digested with DNase I (Invitrogen) and first strand cDNA was synthesized using 1 µg of RNA, oligo-(d)T primer and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

For the analysis of SR gene expression in the corresponding *sr* mutant background, total RNA and cDNA synthesis were performed as described above, using rosette leaves as starting material. The expression of SR genes was assessed by semi-quantitative reverse transcription PCR (RT-PCR), using the Paq5000 DNA polymerase (Agilent). Primers flanking the insertion were used to determine the SR gene's expression in the corresponding mutants, and the *ACTIN-2* (*ACT2*) housekeeping gene was used as a reference gene.

### **4. Quantitative real-time RT-PCR (RT-qPCR)**

RT-qPCR was performed in 384-well reaction plates using the QuantStudio™ 7 Flex Real-Time PCR System. The reactions were prepared in a total volume of 10 µl containing 2.5 µl cDNA (diluted 1:5), and 7.5 µl of Luminaris Color HiGreen High Rox qPCR Master Mix (Thermo Scientific), containing 300 nM of each gene-specific primer. No-template controls (NTCs) were included for each gene in both primer efficiency tests and comparative gene expression analyses. The cycle conditions were set as follows: initial template denaturation at 95°C for 5 min, 40 cycles of 95°C for 30 s, 60°C for 20 s and 72°C for 30 s, followed by a step of gradually increasing temperature to determine the melting point of each primer pair and to confirm the specificity of the amplified products. *ELONGATION FACTOR 1 ALPHA* (*EF1α*) was used as the endogenous reference gene.

For each condition tested, two independent biological and technical replicates were performed, as described by Remy *et al.* [63]. Data were processed using Q-Gene [64]. Statistical analyses were performed using Student's t-test, where a two-sided p-value < 0.05 was accepted to indicate statistical significance.

## 5. Phenotypical analyses

Plants of different genotypes were sown and grown simultaneously under identical conditions. Seeds from fully matured siliques of dehydrated plants of the same age were collected and stored in the dark at room temperature. All assays were performed using seeds from comparable lots stored for at least two to three weeks.

For seed germination assays, seeds were sterilized and water imbibed as described previously. After stratification, 100-200 seeds of each genotype were sown in triplicate in Petri dishes containing MS medium supplemented or not with the appropriate concentrations of NaCl (Sigma), mannitol (Sigma) or ABA (Sigma). Plates were then placed in a controlled environment reach-in chamber under long-day photoperiod (16 h light, 22°C/8 h dark, 18°C) and 60% relative humidity under standard light ( $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) or low light ( $40 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) conditions.

Germination (defined as the protrusion of the radicle from the seed coat), cotyledon greening and cotyledon expansion were scored every day after transfer to the growth chamber. Cotyledon greening and expansion rates were expressed as the percentage of the total germinated seeds. Average percentages were calculated with standard error (SE) of the triplicates.

## 6. Generation of transgenic lines

cDNAs of the different *SR34* splice variants were amplified using the high fidelity Platinum Taq DNA polymerase (Invitrogen) using primers that allowed the introduction of *PacI* and *SmaI* restriction sites at the 5' and the 3' ends of the PCR product, respectively (**Supplementary Table 1**). The corresponding DNA fragments were first introduced in the pGEM-T vector. After *PacI/SacI* digestion of the pGEM-T vector containing the insert, the *PacI/SacI* fragment was introduced into the pBA002 binary vector, allowing the expression of each *SR34*-eGFP translational fusion under the control of the strong constitutive 35S promoter. The sequence of each construct was verified before plant transformation.

The constructs were then independently introduced into *Agrobacterium tumefaciens* for floral dip transformation [65] of wild type (Col-0) or *sr34-1* mutant plants. T1 transformants were selected on BASTA-containing medium. Parts of the T1 transformant roots were used for microscopical analyses.

After transformation of the different *SR34*-eGFP constructs in *A. tumefaciens* strain GV3101, we transiently expressed in *N. benthamiana*, these translational fusions by agroinfiltration, as described in Voinnet *et al.* [66].

## 7. Microscopy

Confocal and fluorescence images were acquired on a Leica SP5 confocal, using 40x or 63x oil immersion objectives, and in a commercial Leica High Content Screening microscope, using a 20x

objective, respectively. Excitation/detection wavelengths used to detect fluorescence were 488/500–550 nm for GFP, 360-405/420-470 nm for DAPI, and 543/650-710 nm for chlorophyll autofluorescence. For eGFP, fluorescence detection parameters (such as laser intensity, offset, gain and pinhole settings) were set so the fluorescence signal emitted by the eGFP fusion constructs in *N. benthamiana* leaves and *Arabidopsis* root tips was just below the saturation threshold.

## 8. Water loss assay

Water loss rates from leaves detached from wild-type (Col-0) and mutant plants were determined by using four samples of two rosette leaves per genotype and measuring the fresh weight (FW) at several time points over a 3-h period, during which the leaves were subjected to the drying atmosphere.

# III. RESULTS

## 1. Functional analysis of the *Arabidopsis* *SCL33* and *SCL30a* genes during early seedling development

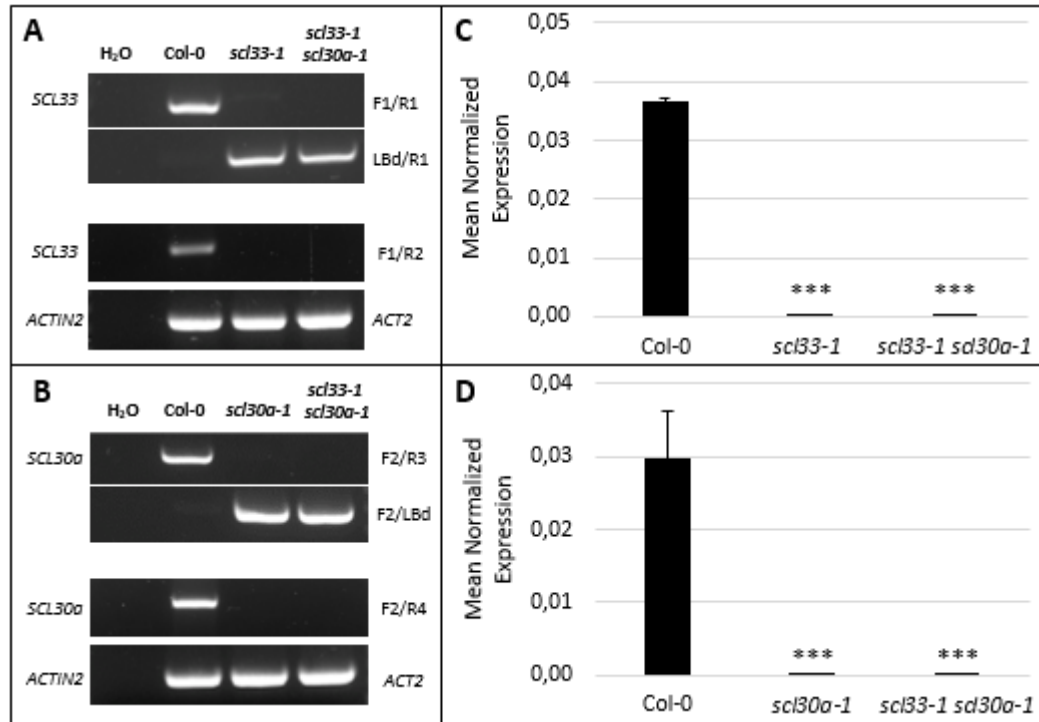
### 1.1. Isolation of the *Arabidopsis* *scl33-1*, *scl30a-1* and *scl33-1 scl30a-1* mutants

To initiate the functional characterization of the *SCL33* gene, mutant lines for *SCL33* (SALK\_058566) and *SCL30a* (SALK\_058566), carrying a T-DNA insertion in the genes' seventh and second exons, respectively (**Supplementary Figures 1 and 2**), were obtained. These lines were then crossed to produce an *scl33-1 scl30a-1* double mutant.

Plants carrying the T-DNA insertion were checked by PCR-based genotyping using primers specific for *SCL33*, *SCL30a* and the left border of the T-DNA (**Fig. 1.1.A and 1.1.B**) (**Supplementary Table 1**). This method allowed us to identify homozygous lines for each insertion. PCR products including the genomic DNA (gDNA) and T-DNA junction were then sequenced in order to confirm the exact site of the insertion.

Semi-quantitative RT-PCR analyses of *SCL33* and *SCL30a* expression levels in the *scl33-1* and *scl30a-1* mutants, respectively, and of both genes in *scl33-1 scl30a-1* double mutant plants revealed no detectable expression when using primers flanking the T-DNA insertion site (**Supplementary Table 1**), suggesting that the genes of interest are at least strikingly downregulated in the corresponding mutant lines (**Fig. 1.1.A and 1.1.B**). The expression levels of *SCL33* and *SCL30a* in mutant rosette leaves was also quantified by quantitative real-time RT-PCR (RT-qPCR) using primers flanking the T-DNA insertion for each mutant line (**Supplementary Table 1**). As shown in **Figures 1.1.C and 1.1.D**, the expression of

*SCL33* and *SCL30a* was barely detectable in plants carrying the T-DNA insertion in each of these genes, suggesting that *scl33-1*, *scl30a-1*, and *scl33-1 scl30a-1* are true loss-of-function mutants. The possibility that truncated transcripts are expressed at wild type levels cannot be excluded but, even in this case, it is expected that these mutants encode non-functional SR proteins.



**Figure 1.1 Isolation and molecular characterization of the *scl33-1*, *scl30a-1* and *scl33-1 scl30a-1* mutants**

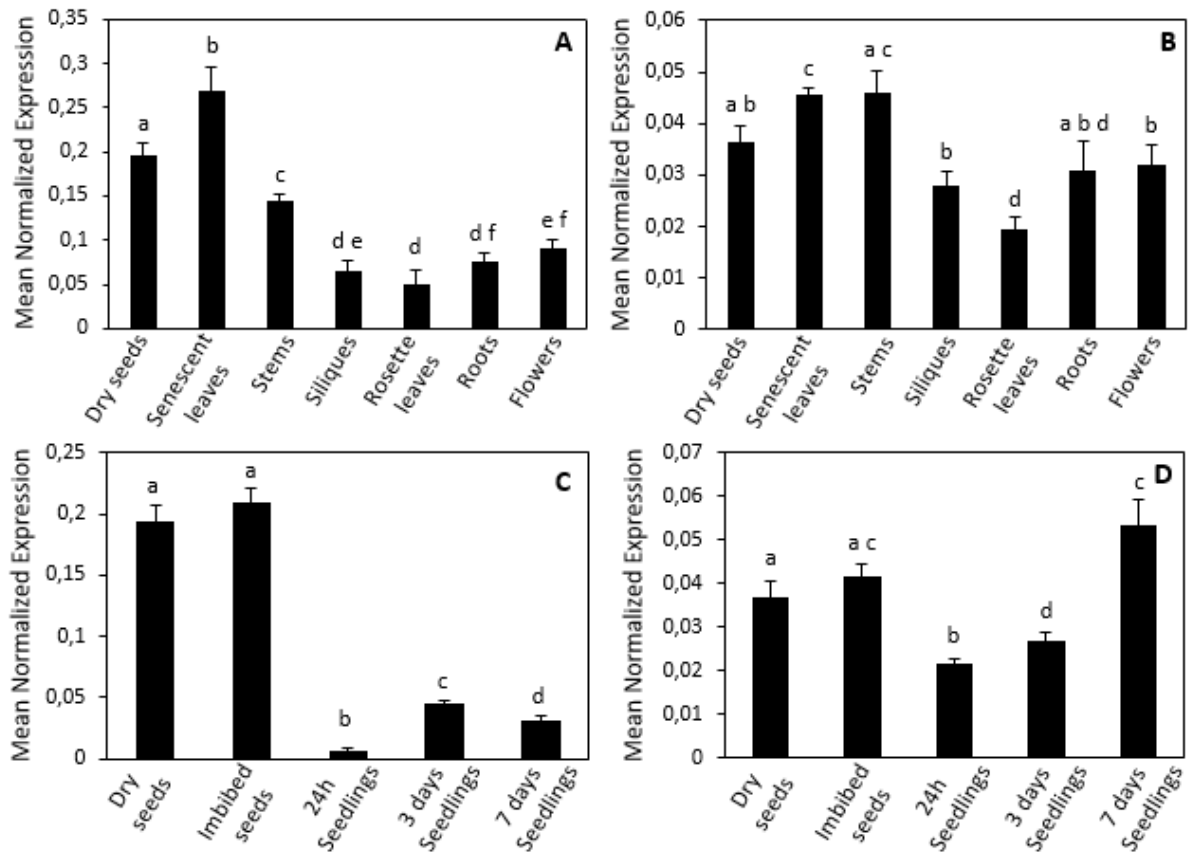
(A-B) PCR-based genotyping of the T-DNA insertion mutant lines *scl33-1*, *scl30a-1* and *scl33-1 scl30a-1*. The disruption of the *SCL33* (A) and *SCL30a* (B) genes was assessed using gene-specific primers flanking the insertion (F1/R1 for *SCL33*; F2/R3 for *SCL30a*). Presence of the T-DNA insertion was assessed using a primer annealing to the left border of the T-DNA (LBd) in combination with the appropriate gene-specific primer. Disruption of the *SCL33* (A) and *SCL30a* (B) transcripts was confirmed by RT-PCR using gene-specific primers annealing to the coding region flanking the T-DNA (F1/R2 for *SCL33*; F2/R4 for *SCL30a*). The *Actin-2* gene was used as a loading control. (C-D) Quantitative real-time RT-qPCR analysis, using primers flanking the T-DNA insertion in the *SCL33* (C) and the *SCL30a* (D) genes, in the wild type (Col-0) and *scl33-1*, *scl30a-1* and *scl33-1 scl30a-1* mutant backgrounds. *EF1α* was used as a reference gene. The results are shown for two independent biological and technical replicates and the values represent means  $\pm$  SE ( $n = 4$ ). Asterisks indicate significant differences (\*\*\*)  $p < 0.001$ ; Student's t-test) from the corresponding wild type.

## 1.2. Functional characterization of the *Arabidopsis scl33-1*, *scl30a-1* and *scl33-1 scl30a-1* mutants

### 1.2.1. Expression patterns of *SCL33* and *SCL30a* during plant development

To obtain clues on the biological role(s) of the *SCL33* and *SCL30a* proteins and aid in the phenotypic analyses of the corresponding mutants, we examined the expression profiles of *SCL33* and *SCL30a* in different tissues/organs and development stages of *Arabidopsis* using RT-qPCR. As shown in **Figures**

**1.2.A** and **1.2.B**, these genes were ubiquitously expressed in the different vegetative and floral tissues analyzed, suggesting a general role for these proteins in plant development. During early developmental stages, we noticed a higher expression level of *SCL33* in seeds (dry and imbibed seeds) in comparison to the other stages analyzed (**Fig. 1.2.C**), while the *SCL30a* gene showed a more stable expression level (**Fig. 1.2.D**). Given the previously described role for *SCL30a* during germination and early seedling development, and the high expression level of *SCL33* in seeds, we decided to focus our study of these two genes during germination and post-germination events.

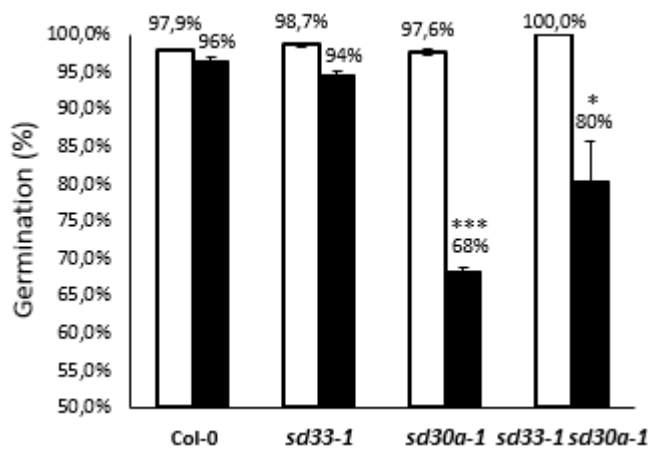


**Figure 1.2 Expression profile of the *SCL33* and *SCL30a* genes in different tissues and during early development**  
Quantitative real-time RT-qPCR analysis of total transcript levels of *SCL33* (A and C) and *SCL30a* (B and D) in different tissues/organs and developmental stages of wild type (Col-0) plants, using *EF1α* as a reference gene. Results are from two independent biological and technical replicates and values represent means  $\pm$  SE ( $n = 4$ ). Different letters indicate statistically significant differences ( $p < 0.05$ ; Student's t-test).

### 1.2.2. Phenotypic characterization of the *scl33-1*, *scl30a-1* and *scl33-1 scl30a-1* mutants

Unpublished work from the PMB lab has shown that the *SCL30a* protein is a negative regulator of the ABA pathway, relying on this hormone to regulate seed-specific traits, such as size and dormancy, as well as germination under high salt and osmotic conditions (Carvalho & Richardson *et al.*, manuscript in preparation). Indeed, the *scl30a-1* loss-of-function mutant exhibits enhanced seed dormancy and hypersensitivity in the response to ABA-related stresses. We therefore asked whether its close paralog, *SCL33*, could share the same functions as *SCL30a* during ABA-related stress responses.

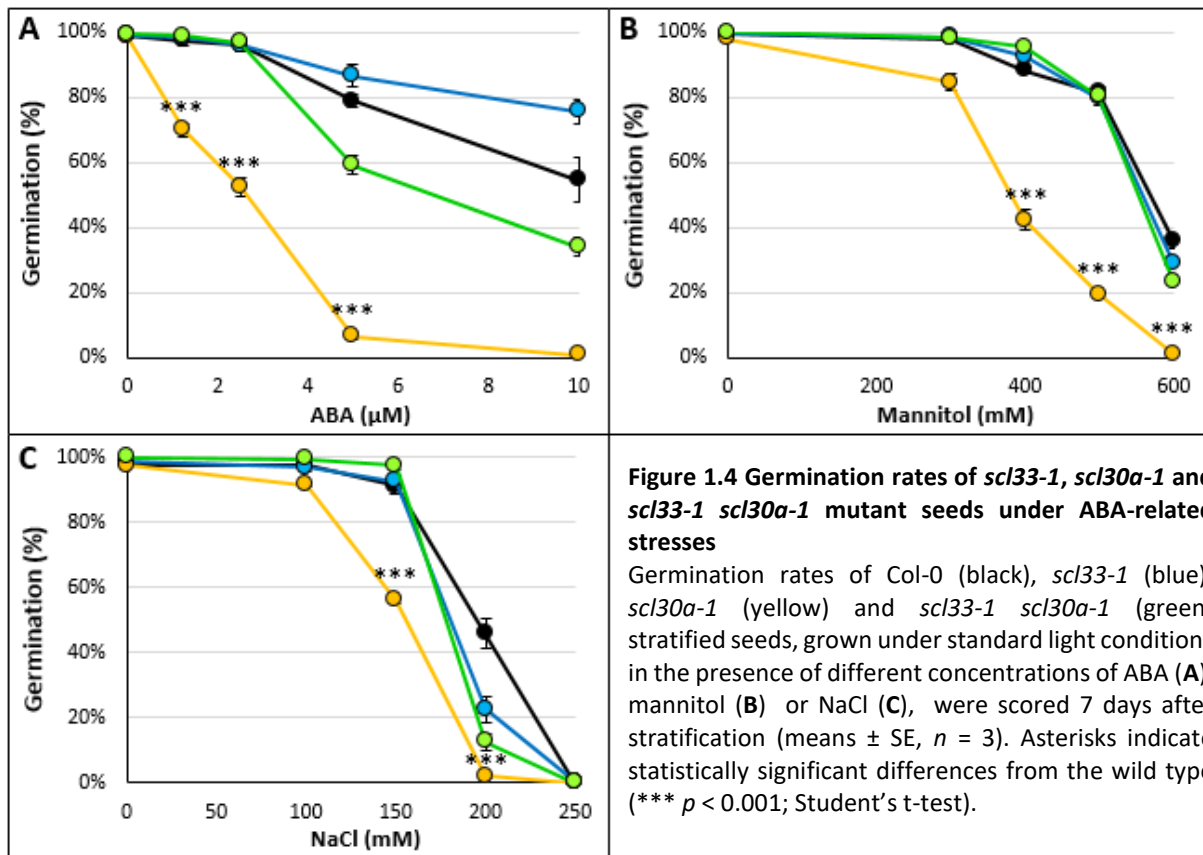
To this end, we first assessed the seed dormancy of the *sc/33-1*, *sc/30a-1* and *sc/33-1 sc/30a-1* mutants (**Fig. 1.3**). Seeds that had been stratified (to break dormancy) were used as positive controls for each genotype to ensure that the observed dormancy phenotypes were not due to any seed viability related problem. Consistent with previous results from our lab (Carvalho & Richardson *et al.*, manuscript in preparation), after 7 days of germination under control conditions, *sc/30a-1* seeds exhibited a strong impairment in germination rates when compared to non-stratified wild type seeds. By contrast, germination of *sc/33-1* mutant seeds was unaffected, suggesting that dormancy is not affected by the lack of a functional *SCL33* gene. Surprisingly, we found that germination of the *sc/33-1 sc/30a-1* double mutant was significantly higher than that of the *sc/30a-1* single mutant. Thus, loss of *SCL33* function can partially rescue the hyperdormant phenotype of *sc/30a-1* mutant by negatively regulating seed dormancy. Together, these results point to opposite roles between *SCL33* and *SCL30a* during seed dormancy, where *SCL33* would act as a positive regulator of this process.



**Figure 1.3 Germination rates of *sc/33-1*, *sc/30a-1* and *sc/33-1 sc/30a-1* mutant seeds**

Germination of freshly-harvested wild type (Col-0), *sc/33-1*, *sc/30a-1* and *sc/33-1 sc/30a-1* seeds scored after 7 days of incubation under control conditions with (white bars) or without (black bars) seed stratification (means  $\pm$  SE,  $n = 3$ ). Asterisks indicate significant differences (\*  $p < 0.05$ ; \*\*\*  $p < 0.001$ ; Student's t-test) from the corresponding non-stratified wild type.

We then evaluated seed germination and early seedling development of the *sc/33-1*, *sc/30a-1* and *sc/33-1 sc/30a-1* mutants under different ABA-related stresses. For this, germination of wild type and mutant seeds was assessed under ABA, salt (NaCl), and drought (mimicked by high concentrations of mannitol) stresses. Consistent with previous observations in the lab, we detected a hypersensitive phenotype for the *sc/30a-1* mutant, with germination being strongly altered in response to all stresses tested (**Fig. 1.4**). By contrast, no significant differences were observed between wild type seeds and the *sc/33-1* mutant. Interestingly, the strong *sc/30a-1* stress hypersensitivity observed during germination was suppressed in the *sc/33-1 sc/30a-1* double mutant. Again, this result suggests that the *SCL33* and *SCL30a* genes play opposite roles during germination of the seed, with *SCL33* being a negative regulator of seed germination under stress conditions. Furthermore, as the *sc/33-1* single mutant exhibits the same germination pattern under stress conditions as wild type seeds, we conclude that the function of *SCL33* becomes relevant only when *SCL30a* is altered or absent.

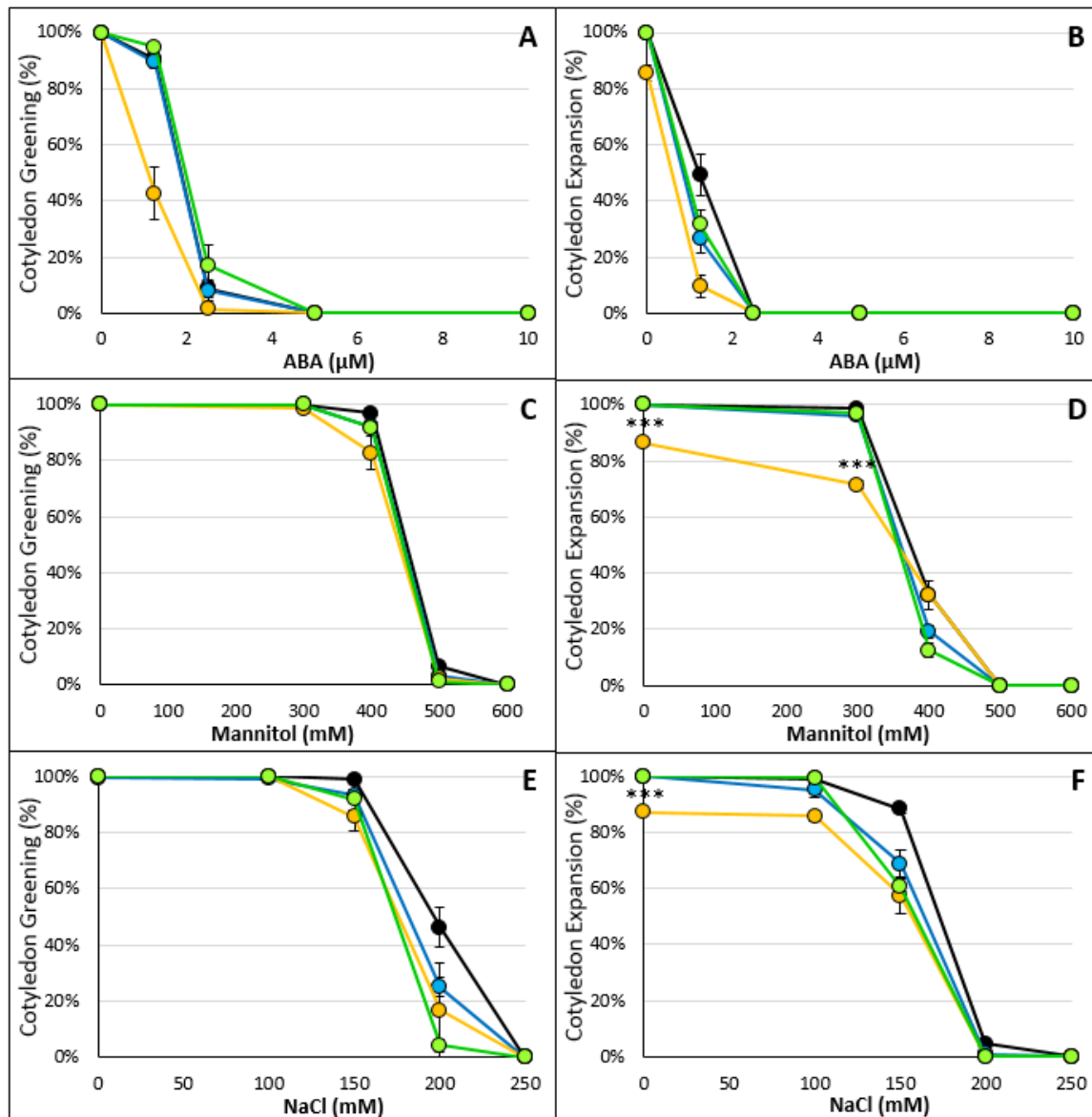


As ABA-related stresses are also known to affect later stages of seedling development, such as cotyledon development, we also compared this developmental processes in wild type and *scl33-1*, *scl30a-1* and *scl33-1 scl30a-1* seedlings. As shown in **Figure 1.5**, only the *scl30a-1* mutant showed significant differences in cotyledon greening or expansion from the wild type under the different treatments. Indeed, this mutant was slightly more sensitive ( $p < 0.05$ ) than the other mutant lines during the majority of the treatments.

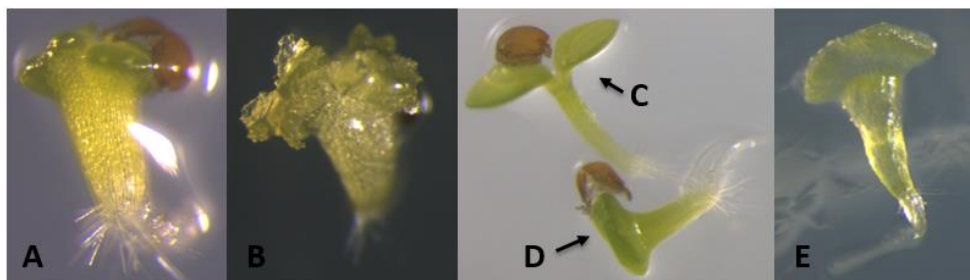
Interestingly, we noticed a striking difference in cotyledon expansion of the *scl30a-1* mutant under control conditions and in response to all the stress treatments for 9% of the *scl30a-1* mutant population (**Fig.1.5** and **1.6**). Although this population showed normal cotyledon greening, cotyledon shape and development was altered, giving rise to an unorganized mass of cells similar to plant calli. These seedlings were able to survive at least one month after germination under control conditions.

Germination, cotyledon greening and cotyledon expansion assays were conducted on seeds from five consecutive generations and similar results were obtained.





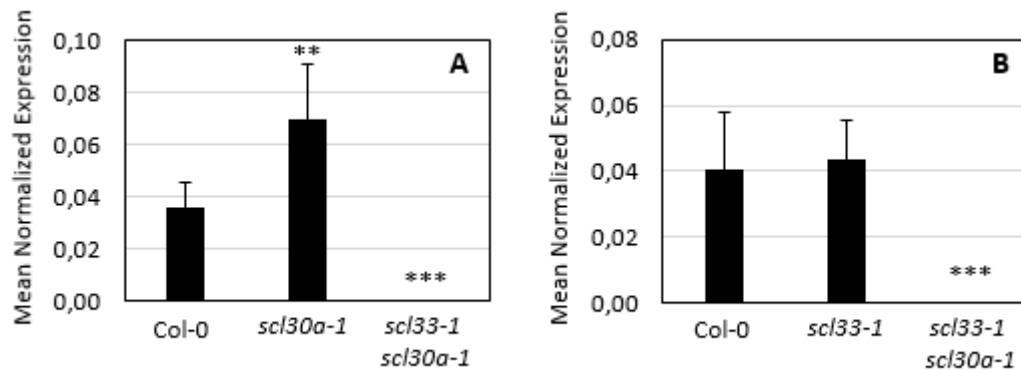
**Figure 1.5 Cotyledon greening and expansion rates of *scl33-1*, *scl30a-1* and *scl33-1 scl30a-1* mutant seeds**  
Cotyledon greening (A, C and E) and cotyledon expansion (B, D and F) rates of Col-0 (black), *scl33-1* (blue), *scl30a-1* (yellow) and *scl33-1 scl30a-1* (green) stratified seeds, grown under standard light conditions in the presence of different concentrations of ABA (A-B), mannitol (C-D) and NaCl (E-F) (means  $\pm$  SE,  $n = 3$ ). All three parameters were scored 7 days after seed stratification. Asterisks indicate statistically significant differences from the wild type (\*\*\*)  $p < 0.001$ ; Student's t-test).



**Figure 1.6 Part of the *scl30a-1* population is incapable of proper development under control conditions**  
Representative images of abnormal *scl30a-1* seedlings 4 (A and D) and 30 (B and E) days after stratification and transfer to light. A 4-day old normally developed seedling (C) is shown as representative of the rest of the population

Given the ABA-related phenotypes of *scl30a-1* mutant seeds, and because ABA is also known to play a key role in vegetative tissues in the regulation of stomatal apertures [6], we next asked whether the *Arabidopsis* SCL33 and SCL30a SR proteins would play an ABA-dependent role in stomatal movements and hence in the control of leaf water loss under drought stress conditions. To address this, we measured the transpiration rates of detached rosette leaves from wild type plants and the three mutant lines. As shown in **Supplementary Figure 3**, we could not detect any significant ( $p > 0.05$ ) changes in water loss rates for the mutant lines. This observation suggests that neither the *SCL30a* nor *SCL33* genes are involved in the regulation of stomatal movements in response to stress.

Thus far, we have been able to confirm the previously described phenotype of the *scl30a-1* mutant during germination under stress conditions. On the other hand, we have found that disruption of a close paralog of *SCL30a*, *SCL33*, does not seem to affect seed dormancy, germination or early development of *scl33-1* mutant plants. In fact, our results point to a role for *SCL33* only in the absence of *SCL30a* function. This suggested that there are compensatory mechanisms between the two genes, whereby one gene directly or indirectly controls the expression/activity of the other. To test this hypothesis, we determined the expression levels of *SCL33* (**Fig. 1.7.A**) and *SCL30a* (**Fig. 1.7.B**) genes in wild type and mutant backgrounds by RT-qPCR. As shown in **Figure 1.7.A**, we detected a two-fold induction of *SCL33* transcript levels in the *scl30a-1* mutant background. By contrast, expression of the *SCL30a* gene was not altered in the *scl33-1* mutant (**Fig. 1.7.B**). These results suggest that *SCL30a* directly or indirectly regulates *SCL33* expression.



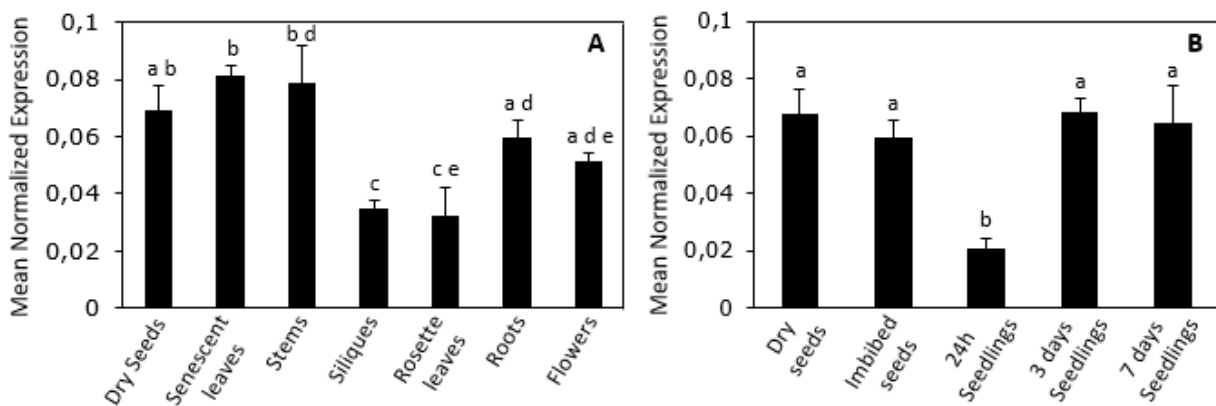
**Figure 1.7 SCL33 and SCL30a expression in the *scl33-1*, *scl30a-1*, and *scl33-1 scl30a-1* mutants**

Quantitative real-time RT-qPCR analysis of total transcript levels of *SCL33* (A) and *SCL30a* (B) in wild type (Col-0), *scl30a-1*, *scl33-1*, and *scl30a-1 scl33-1* mutant rosette leaves, using *EF1α* as a reference gene. Results are from two independent biological and technical replicates and values represent means  $\pm$  SE ( $n = 4$ ). Asterisks indicate statistically significant differences from the wild type (\*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ ; Student's t-test).

## 2. Functional characterization of the *Arabidopsis* *SR34* gene during early development

### 2.1. Expression and splicing pattern of *SR34* during plant development

As a first approach to characterize the functional role of the *Arabidopsis* *SR34* gene, we analyzed its developmental and tissue-specific expression profile by RT-qPCR. As seen in **Figure 2.1**, the *SR34* gene is ubiquitously expressed in *Arabidopsis thaliana* vegetative tissues and during early developmental, suggesting a global role for this gene throughout plant development.



**Figure 2.1 Expression profile of the *SR34* gene**

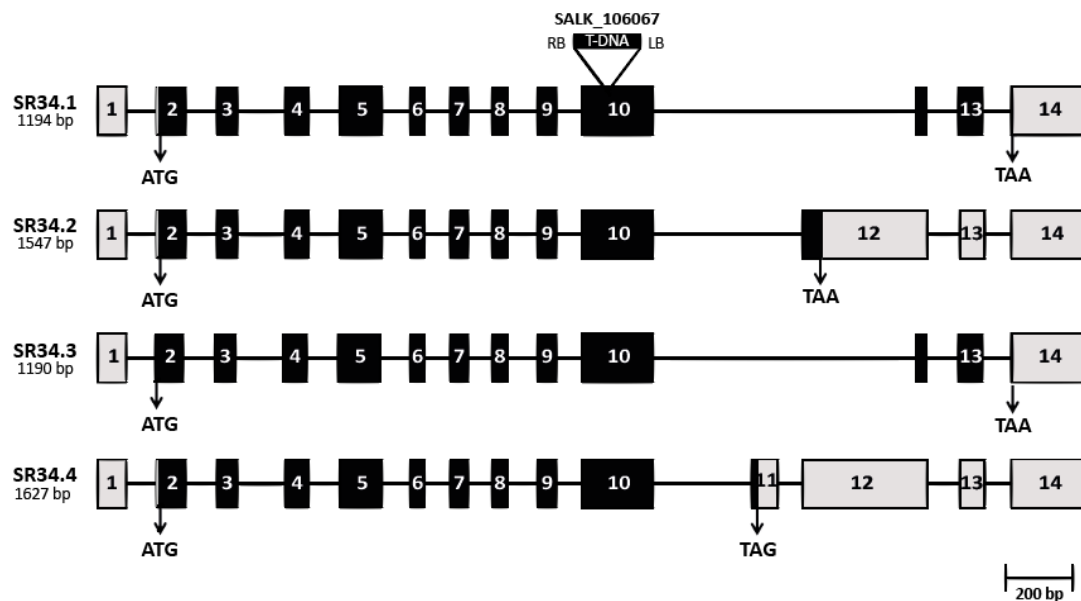
Quantitative real-time RT-qPCR analysis of total *SR34* transcript levels in different tissues (**A**) and early development stages (**B**) of wild type (Col-0) plants, using *EF1α* as a reference gene. Results are from two independent biological and technical replicates and values represent means  $\pm$  SE ( $n = 4$ ). Different letters indicate statistically significant differences ( $p < 0.05$ ; Student's t-test).

Both animal and plant pre-mRNAs encoding SR proteins are themselves particularly prone to alternative splicing. This prompted us to examine the splicing pattern of the *SR34* gene. Our RT-PCR analyses in both rosette leaves and young seedlings resulted in two distinct bands of different relative intensities. Sequencing these two gel bands revealed that one contains two different already annotated ([www.Arabidopsis.org](http://www.Arabidopsis.org)) splice variants (*SR34.1* and *SR34.3*), which differ only in four base pairs in the 5'UTR due to an alternative 3' splice site event. The higher molecular weight band corresponded to a novel splice variant, which we named *SR34.4* (**Fig. 2.2**). Under our experimental conditions, we were unable to detect the annotated *SR34.2* splice variant ([www.Arabidopsis.org](http://www.Arabidopsis.org)). The RT-PCR results obtained here also conflict with previous studies from Reddy and co-workers who reported the presence of six different *SR34* splice variants [61, 67]. Indeed, it is clear that cDNAs from other developmental stages/environmental conditions would need to be tested to enable a comprehensive view of the *SR34* splice variants.

Comparison of the three alternative *SR34*-specific sequences with the genomic fragment revealed that the gene contains at least fourteen exons. The most expressed *SR34.1* transcript arises from

constitutive splicing of the pre-mRNA and, as *SR34.3*, is predicted to encode the full-length SR34 protein. By contrast, *SR34.4* results from a partial intron retention and harbors a PTC.

Taken together, the above results indicate that the *SR34* gene displays ubiquitous expression in embryonic vegetative tissues, being highly expressed during seed imbibition/germination, and produces at least three alternative transcripts. While the *SR34.1* and *SR34.3* mRNAs encode the full-length protein, the *SR34.4* transcript is either a putative NMD target or may be translated into a non-functional or truncated (lacking a part of the SR domain) protein with an altered function.



**Figure 2.2 Schematic diagram of the *Arabidopsis* *SR34* splice variants**

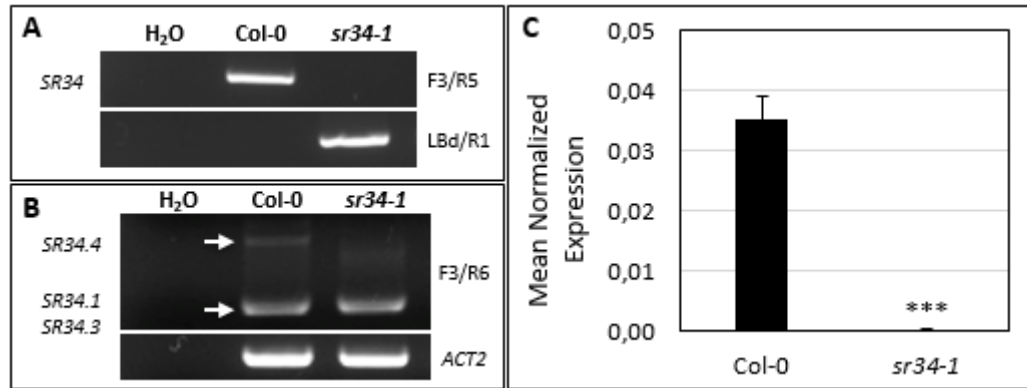
Schematic representation of four *SR34* splice variants (the length of each transcript is indicated). Exons are represented as rectangles and introns as thin lines. Black rectangles represent the coding exons, UTRs are shown in grey. The T-DNA insertion in the SALK\_106067 line is also shown.

## 2.2. Isolation and phenotypic characterization of the *sr34-1* mutant

To initiate the functional characterization of the *SR34* gene, we obtained four T-DNA insertion mutant lines, SALK\_106067, SALK\_102166, SALKseq\_105967 and later SALK\_010894, expected to carry a T-DNA insertion in the gene's tenth exon (SALK\_106067 and SALKseq\_105967), fourth exon (SALK\_102166) or in the 5' UTR (SALK\_010894). The different lines were genotyped using the same PCR-based method as described previously. Of the four independent mutant lines, we were only able to isolate homozygous mutant plants for SALK\_106067 (**Fig. 2.3.A**). This was hence the insertion line used for further functional characterization.

To discern whether the expression of *SR34* was disrupted at the mRNA level, we performed semi-quantitative RT-PCR experiments using gene-specific primers flanking the T-DNA insertion. We detected residual mRNA expression in *sr34-1* when using these primers (**Fig. 2.3.B**). To more precisely

quantify the expression levels of *SR34* in this mutant, RT-qPCR was performed (**Fig. 2.3.C**), using gene-specific primers flanking the T-DNA insertion (**Supplementary Table 1**). Despite residual transcript levels detected in *sr34-1*, these results showed that the *SR34* gene is massively down regulated in this mutant line.

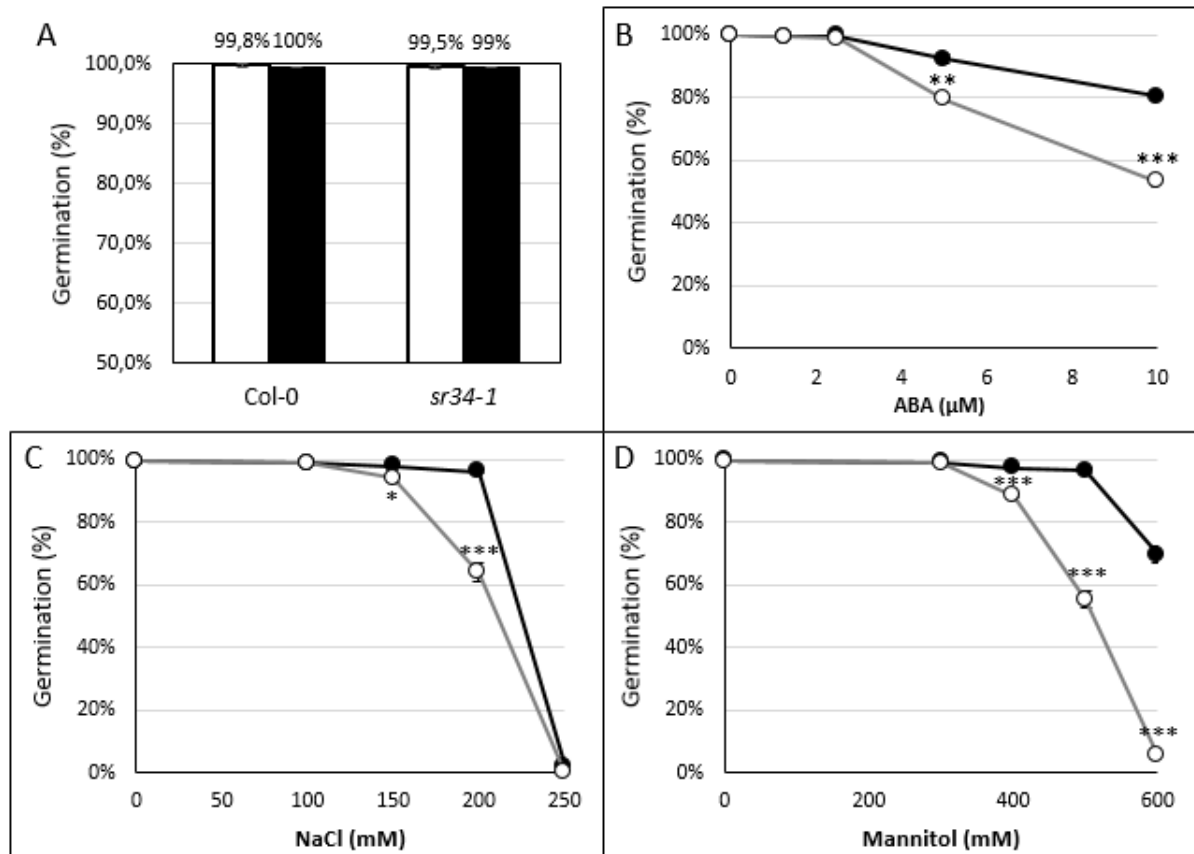


**Figure 2.3 Isolation and molecular characterization of the *sr34-1* mutant**

(A) PCR-based genotyping of the T-DNA insertion mutant line *sr34-1*. Disruption of the *SR34* gene was assessed using gene-specific primers flanking the insertion (F3/R5). Presence of the T-DNA insertion was assessed using a primer annealing to the left border of the T-DNA (LBd) in combination with the appropriate gene-specific primer. (B) Disruption of the *SR34* transcripts was confirmed by RT-PCR using gene specific primers annealing at the coding region, flanking the T-DNA (F3/R6). The *Actin2* gene was used as a loading control. (C) Quantitative real-time qRT-PCR analysis, using primers flanking the T-DNA insertion in the *SR34* gene, in the wild type (Col-0) and the mutant background. *EF1α* was used as a reference gene. The results are shown for two independent biological and technical replicates and the values represent means  $\pm$  SE ( $n = 4$ ). Asterisks indicate significant differences (\*\*\*)  $p < 0.001$ ; Student's t-test) from the wild type.

We then made use of this *sr34-1* loss-of-function mutant to address the role of *SR34* in seed germination and dormancy as well as in ABA-mediated stress responses during germination and early seedling development. As shown in **Figure 2.4**, we did not detect differences in dormancy levels between *sr34-1* mutant and wild type seeds, indicating that the *SR34* protein does not regulate this process.

However, when these mutant seeds were stratified and plated on MS media supplemented with the appropriate concentrations of ABA, NaCl or mannitol, lower germination rates were observed for the *sr34-1* mutant when compared to the wild type. As germination of *sr34-1* mutant seeds was unaffected under control conditions (**Fig. 2.4**), we concluded that *SR34* fulfills a stress-specific function during seed germination. Together, these results demonstrate that the *sr34-1* knockdown mutant is hypersensitive to ABA, as well as to salt and osmotic stress during germination, substantiating a role for this splicing factor as a mediator of ABA-mediated abiotic stress responses during seed germination.



**Figure 2.4 Phenotypic analyses of the *sr34-1* mutant**

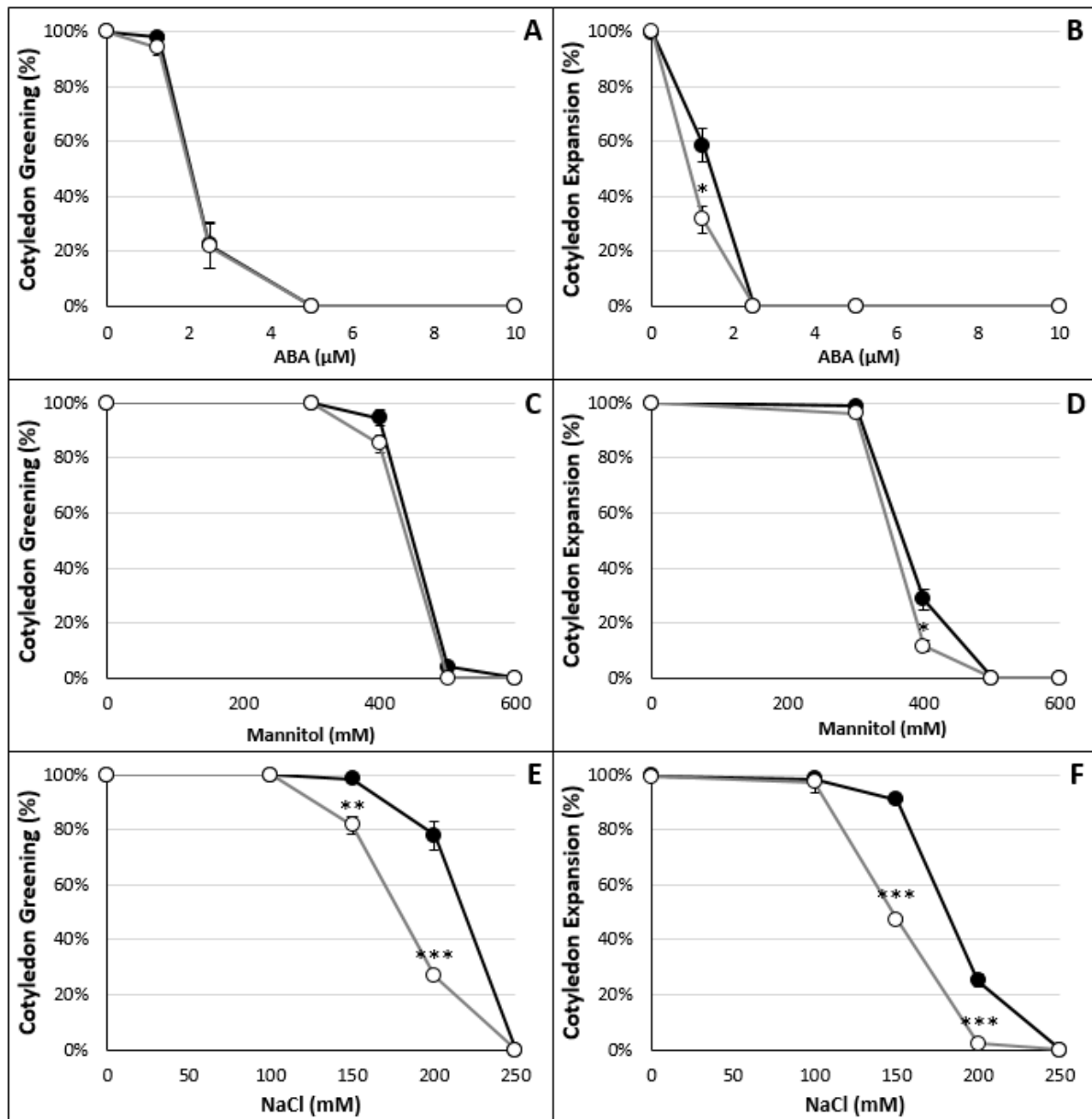
(A) Germination rates of freshly-harvested stratified (white bars) or non-stratified (black bars) Col-0 and *sr34-1* seeds scored after 7 days under control conditions (means  $\pm$  SE,  $n = 3$ ).

(B-D) Germination rates of Col-0 (black) and *sr34-1* (white) stratified seeds grown under standard light conditions in the presence of different concentrations of ABA (B), NaCl (C) or mannitol (D) were scored 7 days after stratification (means  $\pm$  SE,  $n = 3$ ). Asterisks indicate statistically significant differences from the wild type (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ ; Student's t-test).

We next analyzed the phenotype of the *sr34-1* mutant in response to ABA, drought, or salt stress during early seedling development. As shown in **Figure 2.5**, cotyledon greening in the *sr34-1* mutant was impaired only under salt stress (**Fig. 2.5.E**), while no differences from the wild type were observed under ABA and mannitol stresses. However, the salt stress sensitivity was not statistically significant in a second independent generation (results not shown).

A clear phenotype for the *sr34-1* mutant was also observed during the expansion of cotyledons stage, during which this mutant showed a slight impairment when exposed to the three different stress treatments (**Fig. 2.5**). However, only for the ABA treatment this hypersensitivity was observed in both generations analyzed (**Fig. 2.5.B**).

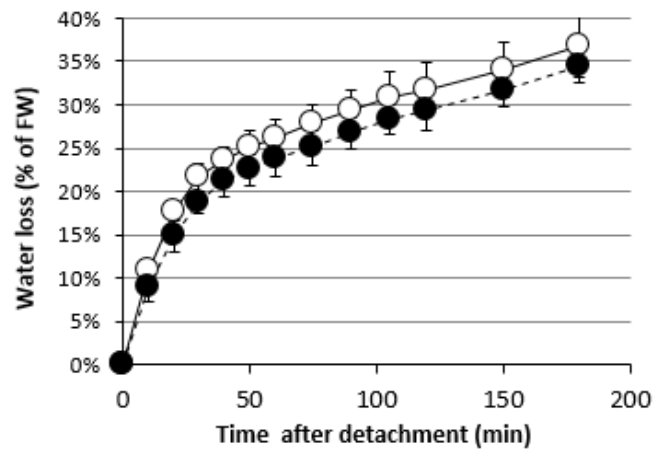
The above results suggest that the *sr34-1* mutant is also impaired in stress responses during early seedling development, though more biological repetitions must be performed to confirm these data.



**Figure 2.5 Cotyledon greening and expansion rates of the *sr34-1* mutant**

Cotyledon greening (A, C and E) and cotyledon expansion (B, D and F) rates in Col-0 (black) and *sr34-1* (white) stratified seeds grown under standard light conditions in the presence of different concentrations of ABA (A-B), mannitol (C-D) and NaCl (E-F) were scored 7 days after stratification (means  $\pm$  SE,  $n = 3$ ). Asterisks indicate statistically significant differences from the wild type (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ ; Student's t-test).

Furthermore, as for the *SCL33/SCL30a* paralogs, we also asked whether the *SR34* gene could play a role in the regulation of stomatal apertures and thus in the control of water loss under drought stress. To address this, we measured the transpiration rates of detached rosette leaves from the wild type and the *sr34-1* mutant. As shown in **Figure 2.6**, we could not detect any significant differences in water loss rates between the mutant and the wild type, suggesting that *SR34* is not involved in an ABA-dependent control of stomatal apertures in response to stress.



**Figure 2.6 Leaf water loss rates of the *sr34-1* mutant**

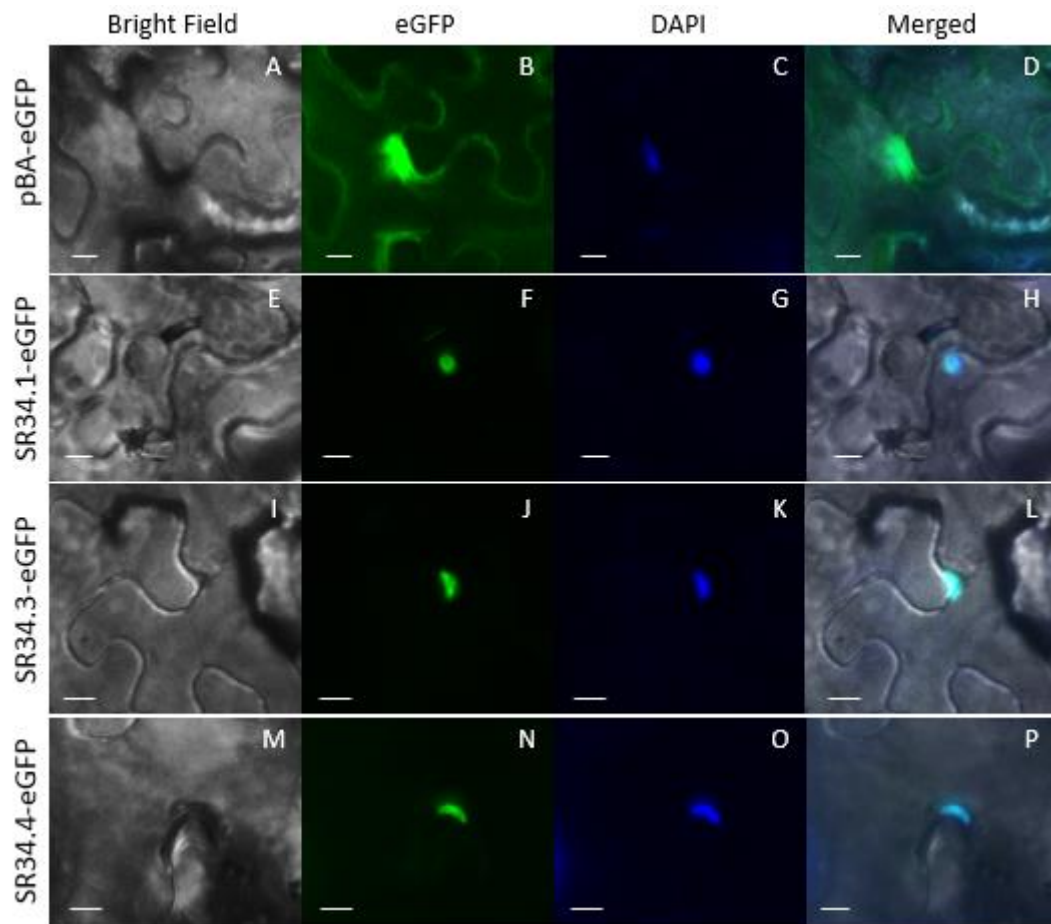
Water loss rates from leaves detached from 5-week old Col-0 (white) and *sr34-1* mutant (black) plants. Four samples of two rosette leaves per genotype were excised from the plant and the fresh weight (FW) determined at several time points over a 3-h period during which the leaves were subjected to the drying atmosphere. Each data point is presented as the mean  $\pm$  SE from four independent experiments ( $n = 4$ ).

### 2.3. Subcellular localization of the *SR34* splice forms

It has recently been demonstrated that in plants, as in animals, SR proteins can shuttle between the nucleus and the cytoplasm [45, 68]. To determine the subcellular localization of the different splice forms encoded by the three *SR34* splice variants, C-terminal eGFP translational fusions of each splice variant were transiently expressed in *N. benthamiana* leaves under the control of the 35S promoter. We used DAPI as a nuclear marker control. While the eGFP protein alone showed a typical nuclear and cytoplasmic localization [69], all three different SR splice forms were found accumulating in the nucleus of *N. benthamiana* cells (**Fig. 2.7**). Although substantiating a role for *SR34* as an mRNA splicing regulator, this result is in contrast with a previous study showing that *SR34* shuttles between the nucleus and the cytoplasm [45].

The three *SR34*-eGFP fusions were also stably expressed in *Arabidopsis thaliana*, and the subcellular localization of these fusions was assessed in roots (**Supplementary Fig. 4**). In contrast with our observations in *N. benthamiana*, in transgenic *Arabidopsis* roots the *SR34* splice forms were localized in both the nucleus and the cytoplasm. This observation could indicate true shuttling activity between the nucleus and the cytoplasm for these proteins, although further experiments should be conducted to confirm this conclusion.





**Figure 2.7 The *Arabidopsis* SR34 splice forms are localized in the nucleus of *N. benthamiana* cells**

Fluorescence microscopy images of *N. benthamiana* leaf epidermal cells transiently expressing the eGFP protein alone (A-D), or the SR34.1-eGFP (E-H), SR34.3-eGFP (I-L), and SR34.4-eGFP (M-P) translational fusions. All protein fusions were expressed under the control of the 35S promoter. DAPI was used as a marker for nuclear localization. The eGFP and DAPI are visualized as green and blue signals, respectively. Scale bars, 10  $\mu$ m.

#### IV. DISCUSSION

Alternative splicing (AS), a versatile means of regulating gene expression and generating proteomic diversity, plays an essential role in plant stress responses. As major modulators of AS, SR proteins constitute very likely candidates as main regulators of these responses. Research on plant SR proteins has been mainly focused on their molecular mode of action, with few functional studies having addressed the role of SR proteins during the response to stress. The work presented in this thesis sheds light on the functional significance of two uncharacterized abscisic acid (ABA)-responsive *Arabidopsis* SR genes — *SR34*, a member of the SR subfamily, and *SCL33*, the paralogous gene of *SCL30a*, both belonging to the plant-specific SCL subfamily. The results obtained here indicate that these two genes play important roles in regulating ABA-related stress responses during early development in *Arabidopsis*.

## 1. *SCL33-SCL30a* genetic relationships during ABA-related stresses

ABA is a key phytohormone for growth, development and stress responses in plants. During seed development, it plays essential roles in maturation and establishment of primary dormancy, thus later regulating seed germination and later germination events [reviewed in 5]. In recent years, various genetic components and different phytohormones have been implicated in the control of seed dormancy and germination [3]. The Plant Molecular Biology (PMB) lab at the *Instituto Gulbenkian de Ciência* (IGC), where the experimental part of this thesis was conducted, has recently found that the *SCL30a* gene acts as a negative regulator of ABA-mediated stress responses. The *sc/30a-1* loss-of-function mutant exhibits stronger seed dormancy and a hypersensitive response to ABA-related stress conditions during germination, with these responses requiring an intact ABA pathway. Moreover, the endogenous ABA content of *sc/30a-1* mutant seeds is unchanged, indicating that the SCL30a RNA-binding protein likely acts in the ABA pathway to repress signaling of the hormone instead of downregulating its biosynthesis (Carvalho & Richardson *et al.* manuscript in preparation).

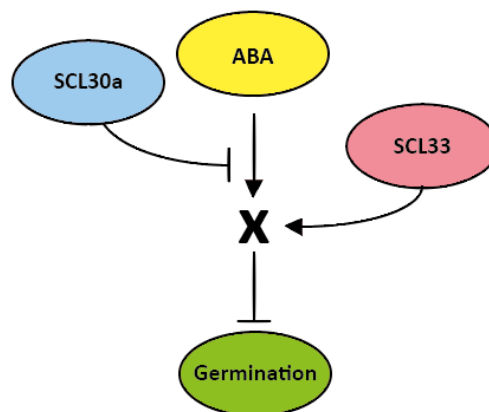
In this study, we addressed the functional role of the close paralog of *SCL30a*, *SCL33*, in the response to ABA-related stresses during early plant development. Unlike *sc/30a-1*, which displays enhanced seed dormancy and hypersensitivity to stress during germination, we could not detect any phenotypes for *sc/33-1* in seeds or during germination events in response to stress. These results suggested that, unlike *SCL30a*, the *SCL33* gene is not involved in the control of plant stress responses during seed germination or early seedling development.

The *sc/33-1* mutant is disrupted in the last coding exon, which encodes part of the RS domain responsible for protein-protein interactions. Although we cannot exclude that a truncated SCL33 protein is produced, the *sc/33-1* mutant is likely to represent a loss-of-function mutant. Indeed, the RS domain of the potential truncated protein would be disrupted and thus defective protein-protein interactions expected in this mutant. In fact, it has been previously reported that a defective RS domain leads to a loss of protein function, through splicing regulation impairment or defective subcellular localization [31, 45, 70]. Future RT-qPCR experiments using primers upstream of the T-DNA insertion should reveal whether a truncated transcript could be produced in the *sc/33-1* line. If a truncated protein is produced, it would also be interesting to assess its subcellular localization to gain clues on whether its splicing activity could be affected.

Here we demonstrate that even if a truncated SCL33 protein is being produced in the *sc/33-1* mutant, it is not able to fulfill the functions of the wild type SCL33 protein, as the *sc/33-1* mutation can rescue the *sc/30a-1* hypersensitivity to ABA-related stresses. In other words, if SCL33 were fully functional in the *sc/33-1* mutant, we would not expect the *sc/33-1 sc/30a-1* double mutant to behave differently from the *sc/30a-1* single mutant. These results point to opposite roles for the *SCL33* and *SCL30a* genes in regulating seed dormancy and germination. Furthermore, as we could only observe a

phenotype for the disruption of *SCL33* when the plant lacks a functional *SCL30a*, we conclude that this gene acts as a positive regulator of ABA-mediated responses only in the absence of *SCL30a*.

From the above-mentioned results, we propose a putative working model in which *SCL30a* and *SCL33* play opposite roles during ABA-responses (**Fig. 3.1**). In this model, the *SCL30a* gene acts indirectly as a negative regulator of the ABA-pathway by repressing the accumulation of yet unidentified inhibitors of germination (X), while *SCL33* functions as an activator of these germination factors. Thus, in the *sc/30a-1* loss-of-function mutant exposed to ABA-related stresses, the unknown germination inhibitors accumulate, and their activation is then promoted by the *SCL33* protein, leading to a reduction in germination. In the *sc/33-1* single mutant, insufficient levels of the germination repressors are present for observation of an obvious phenotype. However, in the *sc/33-1 sc/30a-1* double mutant, *SCL30a* is not present to repress the accumulation of the germination repressors, but neither is *SCL33* to promote their activation, thus maintaining higher levels of germination in the presence of ABA. This model would explain the rescuing of the *sc/30a-1* phenotype observed in the *sc/33-1 sc/30a-1* double mutant. Future identification of the *SCL30a* and *SCL33* target mRNAs should provide important answers on the mode of action of these two proteins during seed dormancy and stress responses.



**Figure 3.1** Schematic model for the mode of action of *SCL30a* and *SCL33*

While the role of AS in ABA-signaling regulation and plant adaptation to stress remains poorly understood, it has been shown that the splicing of several pre-mRNAs is regulated in response to this hormone. For example, the RBM25 splicing factor is known to influence the splicing pattern of several transcripts in response to ABA [71]. Core ABA-signaling genes such as *ABSCISIC ACID INSENSITIVE 3* (*ABI3*) and *HAB1*, also undergo AS in order to regulate seed dormancy and germination [10, 72, 73]. Moreover, *DELAY OF GERMINATION 1* (*DOG1*) and *PHYTOCHROME INTERACTING FACTOR 6* (*PIF6*), which seem to act independently from ABA-pathway, are also alternatively spliced, and this mechanism is crucial for breaking seed dormancy and promoting germination [74, 75]. As first approach to the identification of the molecular targets of the *SCL30a/SCL33* paralogs, it would

therefore be interesting to analyze the splicing pattern of ABA-signaling genes such as those described above in the *scl33-1* and the *scl30a-1* mutants in order to gain mechanistic insight into the mode of action of these splicing factors.

The RT-qPCR results presented here show that both the *SCL30a* and *SCL33* transcripts are ubiquitously expressed in the different plant organs and developmental stages analyzed. These observations are consistent with previous data reporting a general expression for these genes in different *Arabidopsis* tissues [59] and point to a possible more general role during plant development. Regarding early developmental stages, we detected a higher expression level for *SCL33* in seeds (dry and imbibed) in comparison with the other stages analyzed. This may suggest a role for *SCL33* during the seed germination process. To investigate during which specific developmental stages *SCL33* acts and obtain a more detailed expression profile of this gene, it would be interesting to conduct promoter-reporter experiments using transgenic plants stably expressing the  $\beta$ -glucuronidase (GUS) reporter gene under the control of the *SCL33* endogenous promoter.

In response to drought stress, plants synthesize ABA which rapidly induces stomatal closure, thereby reducing transpirational water loss [6]. Despite the fact that both *SCL30a* and *SCL33* are considerably expressed in *Arabidopsis* leaves, results from our water loss assays show that neither the *scl30a-1* nor the *scl33-1* mutants are affected in this process. Furthermore, because the *scl30a-1 scl33-1* double mutant also behaves like the wild type, we can exclude the possibility of compensatory mechanisms between these two genes in the regulation of stomatal movements. Thus, we conclude that *SCL30a* and *SCL33* may be important regulators of stress responses during the very early stages of plant development, but they do not seem to play a role in the ABA signal transduction pathway for stomatal regulation.

## **2. Functional characterization of the *SR34* gene**

In this study, we also used a reverse genetic approach to address the role of the *Arabidopsis SR34* gene during seed germination and early seedling development under stress conditions. To this end, we isolated four *SR34* mutant alleles, and were able to isolate one, *sr34-1*, homozygous for a T-DNA insertion in the tenth exon of the gene. In the other three lines, all the plants analyzed were either heterozygous for the T-DNA insertion or expressed wild type levels of *SR34* mRNA. On the other hand, although *SR34* expression is strongly downregulated in the *sr34-1* mutant, the presence of residual levels of *SR34* transcripts suggests that at least some full-length *SR34* protein can be produced. The above results could reflect the fact that the *SR34* gene is essential for plant growth and development, with only plants expressing this protein, even if only at very low levels, being capable of survival.

Importantly, the data presented here indicate that this gene's downregulation leads to a hypersensitive phenotype during seed germination under ABA-related stresses, pointing to a role for

this splicing factor as a modulator of ABA-mediated stress responses. However, it is important to note that we cannot yet exclude the possibility that the observed phenotype results from disruption of another *Arabidopsis* gene. Further characterization of other *sr34* mutant alleles and/or complementation experiments of the *sr34-1* mutant using the wild type *SR34* gene will verify the involvement of *SR34* in plant stress responses.

We also found that the *sr34-1* mutant seems to be affected by stress during early seedling development, especially during cotyledon expansion in the presence of exogenous ABA. As in our experiments this cotyledon expansion phenotype was only evident at the lowest concentration of ABA tested, further assay using a different range of ABA concentrations should provide clearer evidence for the involvement of *SR34* in this developmental process.

Consistent with previous reports [45, 59], we show here that *SR34* is ubiquitously expressed in *Arabidopsis*, being detected in different plant organs and at various development stages. As with *SCL33*, this observation could suggest a general role throughout plant development. Nevertheless, we could not detect any defects in stomatal aperture for the *sr34-1* mutant. We therefore propose that, like *SCL33*, the *SR34* protein acts specifically in the ABA signaling cascade for early plant development. A more detailed analysis of the *SR34* spatio-temporal expression profile using a *promSR34:GUS* construct would help in the identification of specific tissues and developmental stages where *SR34* may act to control stress responses.

Similarly to the *SCL30a* and *SCL33* gene pair, *SR34* results from a gene duplication and has the *SR34b* gene as a close paralog [55]. Previous reports indicate that *SR34* and *SR34b* share a common expression pattern, although *SR34b* seems to be expressed at much lower levels than *SR34* [45, 55]. Future characterization of *sr34 sr34b* double mutant plants should help elucidate the functional relationships between these two genes during plant stress responses. In addition to *SR34b*, *SR30* and *SR34a* also belong to the same SR gene subfamily, which is characterized by the presence of a highly conserved SWQDLKD motif. It would be also interesting to understand whether these structurally similar proteins act in the same signaling pathways.

In addition, we have shown that the *SR34* gene generates at least three different splice variants, where the *SR34.1* transcript arises from constitutive splicing of the pre-mRNA and, as *SR34.3*, is predicted to encode the full-length *SR34* protein. By contrast, *SR34.4* results from a partial intron retention and harbors a premature termination codon (PTC). This transcript is a putative NMD target, but could be translated into a truncated protein lacking part of the RS domain, which as discussed previously, may lead to a loss of protein function, through splicing regulation impairment or defective subcellular localization [31, 45, 70]. Nevertheless, the subcellular localization of the different protein isoforms potentially encoded by the three *SR34* splice variants had not been characterized prior to the present study.

The full-length SR34 protein was first shown to exhibit a strict nuclear localization in both *Nicotiana tabacum* protoplasts [46] and in *Arabidopsis* transgenic plants [76]. However, recent reports show that in *Arabidopsis* this protein is capable of shuttling between the nucleus and the cytoplasm [45]. In order to gain molecular insight into the mode of action of SR34 and to assess whether AS of SR34 can influence its subcellular localization, we analyzed the subcellular localization pattern of each of the three SR34 splice forms fused to eGFP in *N. benthamiana* and *Arabidopsis thaliana* roots. We found that the different splice forms exhibit the same localization pattern, suggesting that SR34 AS does not play a role in its subcellular localization.

When transiently expressed in *N. benthamiana*, all three SR34 splice forms exhibited a strict nuclear localization. These results are consistent with previous reports in *N. tabacum* protoplasts [46], although we should take into consideration the possibility that *N. benthamiana* does not contain all the required molecular components for a correct shuttling of the SR34 protein. Indeed, it has been demonstrated that different species and organs can affect the subcellular trafficking, structure and qualitative properties of recombinant proteins. For example, the same protein can be differently exported in *N. tabacum* and *A. thaliana* due to different posttranslational modifications [77]. For these reasons, *Arabidopsis* transgenic plants expressing SR34-eGFP constitute a more trustful expression system than a transient heterologous expression assay. Indeed, consistent with a very recent report [45], all SR34 splice forms analyzed in our study exhibited both a nuclear and cytoplasmic localization when stably expressed in *Arabidopsis*. This result corroborates a role for SR34 not only in mRNA splicing but also in post-splicing events. However, the possibility that this SR34-eGFP fusion protein is cleaved in *Arabidopsis* transgenic plants, leading to an eGFP signal similar to that visualized in control plants expressing eGFP alone, cannot be excluded at this point. Additional experiments, such as western blot analyses using anti-eGFP antibodies should be performed in order to confirm the integrity of the SR34-eGFP fusion protein in these transgenic plants.

A key step to unveil the precise mode of action of SR34, and to understand in further detail how this splicing factor regulates ABA responses, is to identify its pre-mRNA targets. Owing to their similar hypersensitive responses to ABA-related stresses, one could expect similar modes of action for the SCL30a, SCL33 and SR34 RNA-binding factors. Future large-scale identification of the molecular targets of SR34, SCL33 and SCL30a will provide crucial answers on the molecular functions of these SR proteins during plant stress responses.

## V. CONCLUSIONS

To conclude, the work described in this thesis provides an important contribution to the elucidation of the biological relevance of SR proteins and AS in plant responses to stress during early development. Using gene expression and subcellular localization analyses combined with reverse genetics in

*Arabidopsis thaliana*, this study provides insights into the *in vivo* functions of two uncharacterized *Arabidopsis* SR genes, *SR34* and *SCL33*, indicating that both act in stress responses mediated by the ABA phytohormone. While *SCL33* appears to positively regulate ABA responses during early plant development, repressing germination under stress conditions, we describe *SR34* as a negative regulator of such stress responses.

Despite the significant amount of functional data gathered on the studied SR genes, further experimental work is required to decipher the exact mode of action of the corresponding proteins. Future RIP (RNA-immunoprecipitation)-seq analyses should allow the identification of common and specific pre-mRNA targets for the *SCL30a*, *SCL33* and *SR34* proteins, thus elucidating the molecular mechanisms governing the function of these three splicing factors in the regulation of ABA-related stress responses.

All SR protein subfamilies share structural similarities and two thirds of the *Arabidopsis* SR proteins include duplicated gene pairs [55], suggesting that SR genes act, at least in part, redundantly. Reverse genetics approaches using single *sr* mutants are therefore likely to mask phenotypes that could reveal important biological roles for SR proteins. Future detailed analyses of SR protein paralogous pairs and other members of the same subfamily, including single and multiple mutant phenotypical characterization, should allow for an exhaustive characterization of the biological functions of plant SR proteins.

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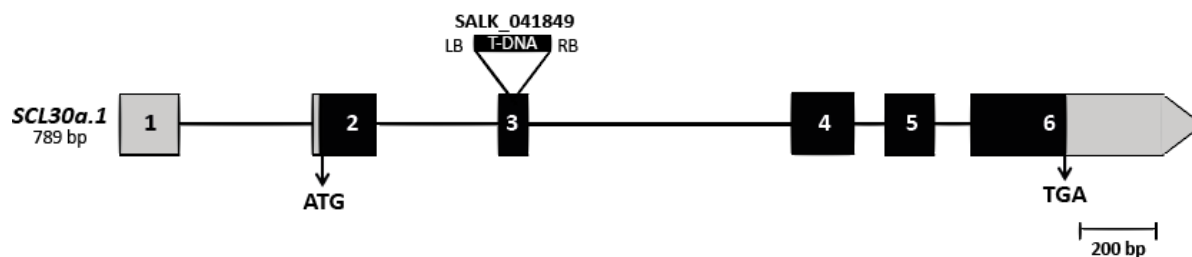
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## VI. ANNEX

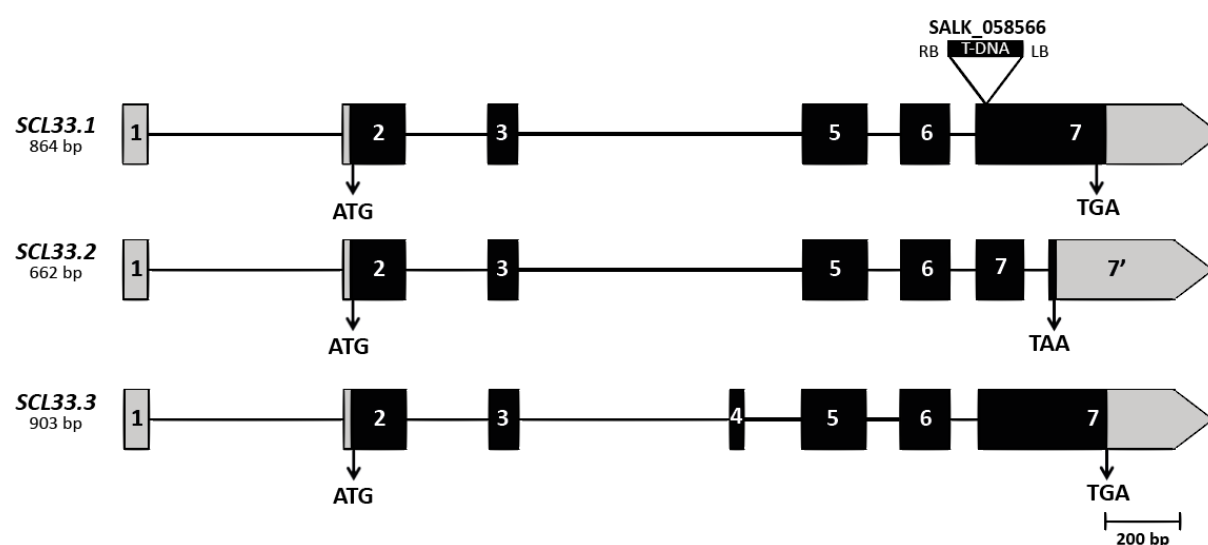
**Table 1. List of the primers used in this study**

Gene Name	Locus ID	Primer sequence (5'-3')	Comment
<b><i>SCL33</i></b>	At1g55310	F1 - TCGGGATAGAAGACGTACTCC	Genotyping
		R1 - GTTCCCCACATGTTCCATAG	Genotyping
		R2 - TATGCTTCTTCTAGGGCTGG	Genotyping
		qF1 - ATCTATCTCGCCCAGGGAAG qR1 - GGCTCTTACCTCTGACTGGAGTT	RT-qPCR RT-qPCR
<b><i>SCL30α</i></b>	At3g13570	F2 - TCTCTTGGTTCGCAACTTACG	Genotyping
		R3 - CCTAAAGTGACTCGAAGAGGG	Genotyping
		R4 - GCCATTATGGGGTGGTGAGCG	Genotyping
		qF2 - CAACCAGTCTCTTGGTTCGC qR2 - TGTCTTGACGGGACCAAAC	RT-qPCR RT-qPCR
<b><i>SR34</i></b>	At1g02840	F3 - GACACAGAGTTTCGAAATGCG	Genotyping
		R5 - GTTCCCACATCCTCTTAGACG	Genotyping
		R6 - GAGATCTTGATCTTGAACGCG	Genotyping
		F4 - <b>GGGTAAATTA</b> AAAAATTGCGGAGGCTGAGAG	<i>SR34</i> cloning
		R7 - <b>TGTCCCGGG</b> ACCTCGATGGACTCCTAGTGTGG	<i>SR34.1</i> and <i>SR34.3</i> cloning
		R8 - <b>TGTCCCGGG</b> TCGAGTCGTATGTAAATCCATATCTTC	<i>SR34.4</i> cloning
		qF3 - GGGAAGATCCTATTCTAAGAGCCG qR3 - GACCTACGTGAAGACTTTGCC	RT-qPCR RT-qPCR
		F - TTTGCAGGAGATGATGCTCCC R - GTCTTTGAGGTTTCCATCTCC	RT-PCR (reference gene)
<b><i>EF1α</i></b>	At5g60390	F - TGAGCACGCTCTTCTTGCTTTCA R - GGTGGTGGCATCCATCTTGTTACA	RT-qPCR (reference gene)
		F - CCCTGATAGACGGTTTTTCGCCC	Genotyping
<b><i>LBd</i></b>	T-DNA		



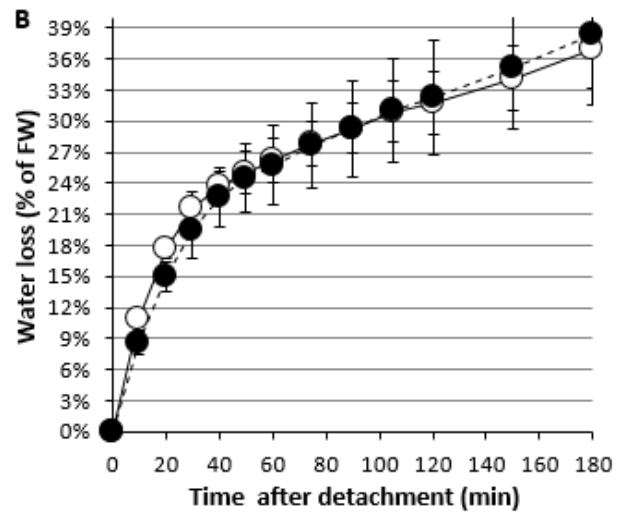
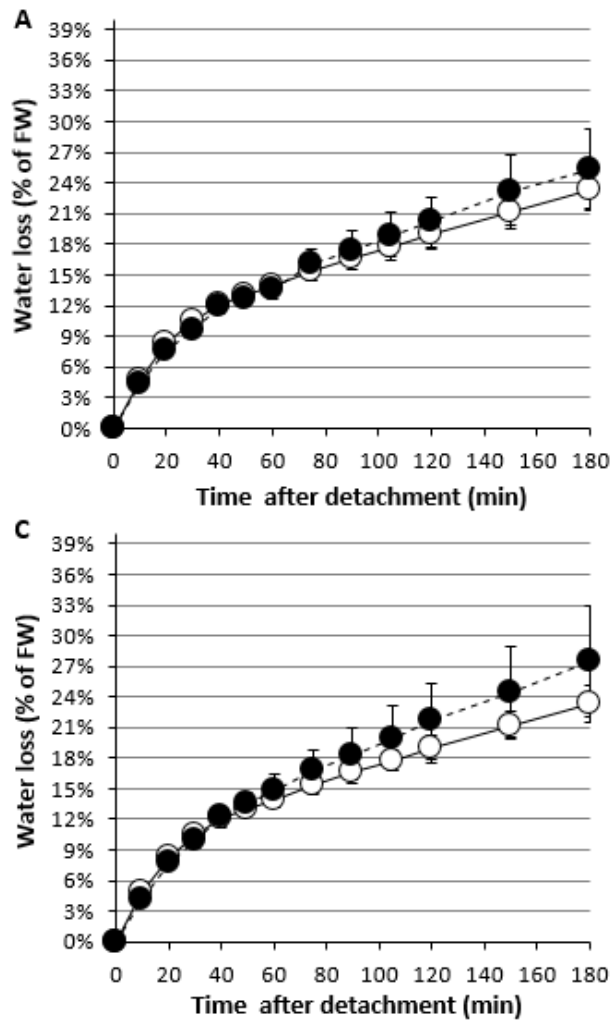
**Supplementary Figure 1. Schematic diagram of the full-length *Arabidopsis SCL30a* transcript**

Schematic representation of the full-length *SCL30a* splice variant (length of the transcript is indicated in bp). Exons are represented as rectangles and introns as thin lines. Black rectangles represent the coding exons, UTRs are shown in grey. The T-DNA insertion in the SALK\_041849 line is also shown.



**Supplementary Figure 2. Schematic diagram of the *Arabidopsis SCL33* splice variants**

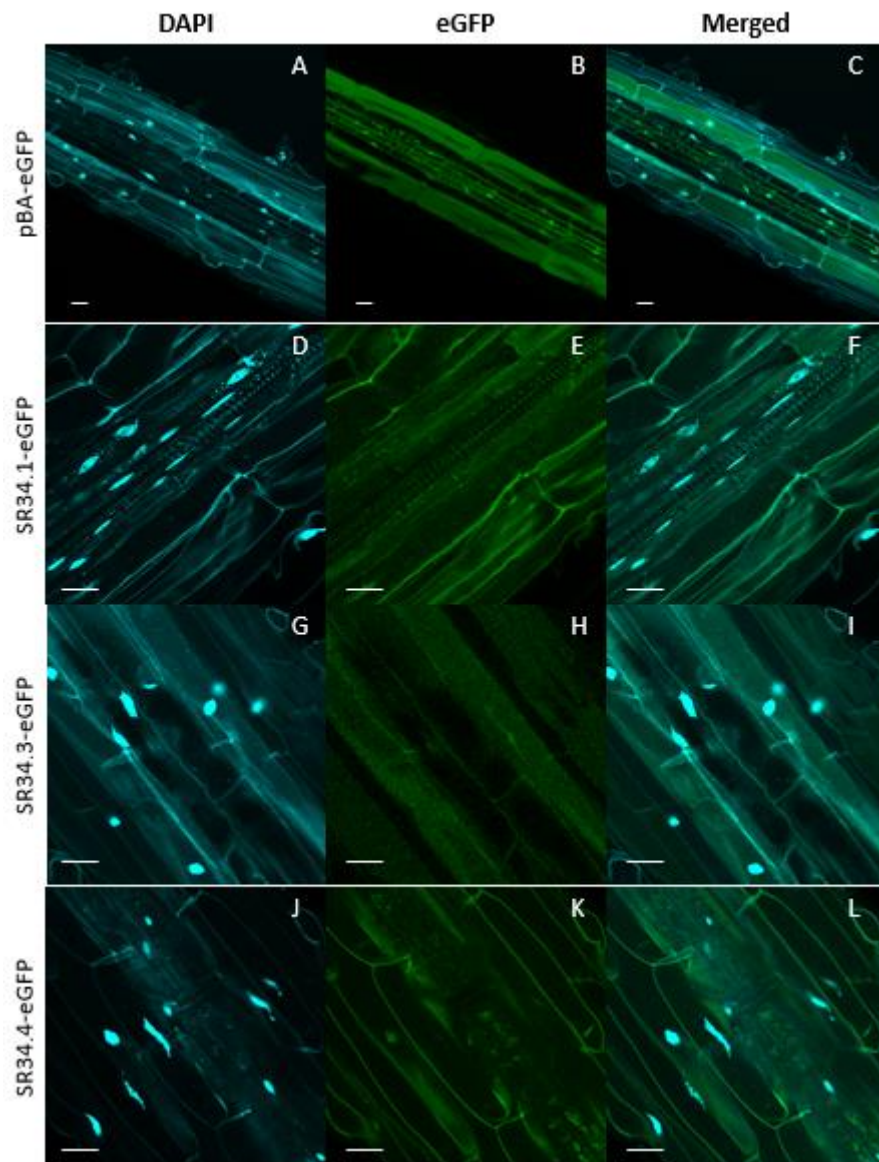
Schematic representation of the *SCL33* splice variants (the length of each transcript is shown in bp). Exons are represented as rectangles and introns as thin lines. Black rectangles represent the coding exons, UTRs are shown in grey. The T-DNA insertion in the SALK\_058566 line is also shown.



**Supplementary Figure 3. Leaf water loss rates in *SCL33* and *SCL30a* mutant lines**

Water loss rates from leaves detached from Col-0 (white) and mutant (black) *scL33-1* (A), *scL30a-1* (B) and *scL33-1 scL30a-1* (C) plants. Four samples of two rosette leaves per genotype were excised from the plant and the fresh weight (FW) determined at several time points over a 3-h period, during which the leaves were subjected to the drying atmosphere. Each data point is presented as the mean  $\pm$  SE from four independent experiments ( $n = 4$ ).





**Supplementary Figure 4. The *Arabidopsis* SR34 splice forms are localized in both the nucleus and the cytoplasm of *Arabidopsis thaliana* root cells**

Confocal microscopy images of root tips of transgenic *Arabidopsis* expressing the eGFP protein alone (A-C) SR34.1-eGFP (D-F), SR34.3-eGFP (G-I) or SR34.4-eGFP (J-L) fusions under the control of the 35S promoter. The eGFP and DAPI are visualized as green and blue signals, respectively. Scale bars, 20  $\mu$ m.